National Library
of Canada

Bibliothèque nationale
du Canada

Canadian Theses Service

Services des thèses canadiennes

Ottawa, Canada
K1A 0N4

CANADIAN THESES

NOTICE

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30.

THIS DISSERTATION
HAS BEEN MICROFILMED
EXACTLY AS RECEIVED

THÈSES CANADIENNES

AVIS

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30.

LA THÈSE A ÉTÉ
MICROFILMÉE TELLE QUE
NOUS L'AVONS REÇUE

NL-339 (r.86/06)

Canada

THE UNIVERSITY OF ALBERTA

Isolation and Characterization of *Campylobacter coli* and *Campylobacter jejuni*

by

Lai-King Ng

©

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF MICROBIOLOGY

EDMONTON, ALBERTA

SPRING, 1987

Permission has been granted to the National Library of Canada to microfilm this thesis and to lend or sell copies of the film.

The author (copyright owner) has reserved other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without his/her written permission.

L'autorisation a été accordée à la Bibliothèque nationale du Canada de microfilmer cette thèse et de prêter ou de vendre des exemplaires du film.

L'auteur (titulaire du droit d'auteur) se réserve les autres droits de publication; ni la thèse ni de longs extraits de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation écrite.

ISBN 0-315-37651-1

THE UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR Lai-King Ng

TITLE OF THESIS Isolation and Characterization of *Campylobacter coli* and *Campylobacter jejuni*

DEGREE FOR WHICH THESIS WAS PRESENTED DOCTOR OF PHILOSOPHY

YEAR THIS DEGREE GRANTED SPRING, 1987

Permission is hereby granted to THE UNIVERSITY OF ALBERTA LIBRARY to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

(SIGNED) Ng Lai King......

PERMANENT ADDRESS:

1037 - 82 Street
EDMONTON, Alberta
T6K 1X5

DATED February 27 1987

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Isolation and Characterization of *Campylobacter jejuni* and *Campylobacter coli* submitted by Lai-King Ng in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Diane E. Taylor
Co-supervisor

Michael E. Stiles
Co-supervisor

Wm Paranchych

W J Page

S E Jensen

Michael P. Doyle
External Examiner

Date 16 February 1987

Abstract

Electron microscopy showed that *C. coli* and *C. jejuni* are morphologically similar. Besides the documented spiral-shaped young cells and coccus-shaped old cells, "donut"-shaped cells were also observed. They were presumed to be an intermediate form between the spiral- and coccus-shaped cells. The correlation between microscopic and plate counts indicated that the coccus-shaped cells were not culturable under the conditions of these studies. Mueller-Hinton (MH) agar (Difco and Oxoid), brucella agar (BBL), and campylobacter agar base (Difco) were found to be equally suitable as basal media for the growth of *C. coli* and *C. jejuni* without added supplements. In subsequent studies, MH agar was used because this medium is also suitable for determining antibiotic suceptibilities of *Campylobacter* spp. Laboratory strains and fresh isolates of *C. coli* were more susceptible than *C. jejuni* to antibiotics used in selective media. This may be a factor in the lower reported incidence of gastroenteritis due to *C. coli*. Limited phenotypic characteristics are available for the taxonomic classification of *Campylobacter* spp., therefore, the DNA base ratio and homology determinations were used to identify atypical *Campylobacter* strains. It was shown that the DNA homology determination procedure could be simplified by a dot-blot hybridization method, which eliminated the need to isolate pure DNA. For strain differentiation of *C. coli* and *C. jejuni*, a combination of biotyping, antibiograms, and

plasmid profiles was shown to be effective. Using a tetracycline-resistant (Tc^R) DNA probe, the mechanism of Tc^R in *C. coli* and *C. jejuni* from animal or human sources was found to be similar. The use of Tc^R probe serves as a model for the development of other DNA probes for diagnostic or detection purposes.

Acknowledgements

I would like to express my gratitude to my two supervisors, Dr. D. E. Taylor and Dr. M. E. Stiles for their support, guidance and patience throughout my Ph.D. program. The helpful advice provided by the members of my advisory committee is greatly appreciated.

I would like to extend my sincere thanks to R. Sherburne, Department of Medical Microbiology, for his assistance with the electron microscopy studies and photography. The assistance, advice and friendship of Elisa Brose, William Yan and Koji Hiratsuka is greatly appreciated. I would also like to acknowledge the departments of Microbiology, Medical Microbiology and Food Science for their support and for providing me with the facilities for my research.

I acknowledge and thank the Alberta Heritage Foundation for Medical Research for the Studentship that supported me throughout my Ph.D. program. The travel support provided by the Alma Mater Fund enabled me to present a poster at the third International Workshop of *Campylobacter* Infection held in Ottawa, 1985.

Table of Contents

Chapter Page

1. Introduction 1

1.1 Bibliography 5

2. Review of Literature 7

2.1 Historical significance of *C. jejuni* and *C. coli* 7

2.2 Incidence and geographical distribution of *Campylobacter* enteritis 8

2.3 Vehicles of transmission and reservoirs of *Campylobacter* 10

2.4 Differential characteristics of *C. jejuni* and *C. coli* 11

2.5 Epidemiological markers for *C. coli* and *C. jejuni* 13

2.6 Antibiotic resistance of *Campylobacter* organisms 14

2.7 Growth of *C. jejuni* and *C. coli* on laboratory media 18

2.8 Isolation methods 19

2.8.1 Filtration 21

2.8.2 Selective media 22

2.8.3 Enrichment media 26

2.9 Concluding remarks 27

2.10 Bibliography 28

3. Morphological forms and viability of *Campylobacter* species studied by electron microscopy 40

3.1 Introduction 40

3.2 Materials and methods 41

3.2.1 Electron microscopy 41

3.2.2 Viability study 42

3.3 Results 43

3.3.1 Scanning electron microscopy 43

3.3.2 Transmission electron microscopy 49

3.3.3 Viability study 54

3.4 Discussion 56

3.5 Bibliography 60

4. Comparison of Basal Media for Culturing *Campylobacter jejuni* and *Campylobacter coli* 62

4.1 Introduction 62

4.2 Materials and methods 63

4.2.1 Preliminary study 63

4.2.2 Stock cultures 64

4.2.3 Cultural conditions 64

4.2.4 Plate counts 65

4.2.5 Data analysis 67

4.3 Results 67

4.4 Discussion 74

4.5 Bibliography 78

5. Inhibition of *Campylobacter coli* and *Campylobacter jejuni* by antibiotics used in selective growth media 81

5.1 Introduction 81

5.2 Materials and methods 83

5.2.1 Cultures 83

5.2.2 Minimal Inhibitory Concentration (MIC) determination 83

5.2.3 Effect of antibiotic combinations used in selective media 84

5.3 Results 87

5.3.1 MIC determinations 87

5.3.2 Effect of antibiotic combinations 89

5.4 Discussion 95

5.5 Bibliography 99

6. Isolation and Characterization of Animal Strains of *Campylobacter* species 103

6.1 Introduction 103

6.2 Materials and methods 105

6.2.1 Reference cultures 105

6.2.2 Sources and isolation of *Campylobacter* spp. 105

6.2.3 Selection of *C. coli* and *C. jejuni* isolates 107

6.2.4 Identification and biotyping of isolates 107

6.2.5 Total genomic DNA isolation and preparation 109

6.2.6 G+C determination 111

6.2.7 DNA homology determination by slot blot hybridization 112

6.2.8 Preparation of ^{32}P-labelled DNA 114

6.2.9 Plasmid analysis 114

6.3 Results 115

6.3.1 Identification and biotyping of isolates 115

6.3.2 Genetic characterization of cultures 119

6.3.3 Plasmid profiles 129

6.4 Discussion 131

6.4.1 Isolation of cultures 131

6.4.2 Identification of isolates from animals 133

6.4.3 Biotyping of isolates 135

6.4.4 Plasmid profiles 135

6.5 Bibliography 137

7. Determination of DNA homology among *Campylobacter* strains using chromosomal DNA probes 142

7.1 Introduction 142

7.2 Materials and methods 143

7.2.1 Bacterial strains 143

7.2.2 Dot blot hybridization 143

7.2.3 DNA-DNA homology determination 145

7.2.4 Preparation of ^{32}P-labelled DNA. 146

7.3 Results 146

7.4 Discussion 154

7.5 Bibliography 156

8. Comparison of the susceptibilities of *Campylobacter coli* and *Campylobacter jejuni* to antibiotics 157

8.1 Introduction 157

8.2 Materials and methods 158

8.2.1 Cultures 158

8.2.2 Antibiotics 159

8.2.3 MIC determinations 160

8.2.4 Effect of selective media 160

8.3 Results 162

8.3.1 MIC determinations 162

8.3.2 Effect of antibiotic combinations 164

8.4 Discussion 168

Bibliography 172

9. Use of DNA probes to study tetracycline-resistance in *Campylobacter* from swine and cattle 174

9.1 Introduction 174

9.2 Materials and methods 175

9.2.1 Bacterial strains 175

9.2.2 Restriction endonucleases 178

9.2.3 Plasmid DNA isolation 178

9.2.4 Plasmid analysis 180

9.2.5 Preparation of ^{32}P-labelled DNA 180

9.2.6 Southern transfer hybridization 182

9.2.7 Minimal Inhibition Concentration (MIC) determination 182

9.2.8 Mating experiments 182

9.2.9 Plasmid curing experiment 183

9.3 Results 183

9.3.1 MIC determination 183

9.3.2 Conditions of dot blot hybridization 185

9.3.3 Dot blot hybridization with 1.8 kb *Hin*cII fragment and *tetM* probes 188

9.3.4 Plasmid analysis and Southern transfer hybridization 188

9.3.5 Restriction endonuclease analysis and Southern transfer hybridization 194

9.3.6 Southern blot hybridization of plasmids with pUA649 203

9.3.7 Southern blot and dot blot hybridization of total genomic DNA with pUA649 probe 203

9.3.8 Mating experiments 209

9.3.9 Curing experiment 212

9.4 Discussion 212

9.5 Bibliography 217

10. Discussion and Conclusions 221

10.1 Bibliography 227

List of Tables

Table Page

2.1 Changes in nomenclature of *C. fetus*, *C. jejuni*, and *C. coli* 9

2.2 Basal media and supplements for growth of *C. jejuni and C. coli* 20

2.3 Combinations of antibiotics used in media for selective isolation of *C. coli* and *C. jejuni* 23

3.1 Microscopic counts of coccoid and spiral forms of *C. coli* and *C. jejuni* compared with plate counts on Mueller-Hinton agar 55

4.1 Composition of basal media used for growth of *Campylobacter* strains 66

4.2 Effects of growth medium and incubation time on viable counts of *C. jejuni* and *C. coli* 69

4.3 Summary of repeated measures analysis of variance for growth response of *C. jejuni* and *C. coli* cultures on different growth media after 48 and 72 h incubation 71

4.4 Summary of two-way (factorial design) analyses of variance for comparison of growth response of cultures inoculated on basal and supplemented media 72

4.5 Log_{10} mean count of *C. jejuni* and *C. coli* plated on basal media without supplements. 73

5.1 Antibiotic composition of selective media used for quantitative comparisons of effects of antibiotics on the growth of *C. coli* and *C. jejuni* 86

5.2 MIC of eight antibiotics against 24 strains of *C. coli* and 6 strains of *C. jejuni* 88

5.3 Susceptibility (MIC) of selected strains of *C. coli* to polymyxin, colistin, rifampicin, cephalothin and novobiocin 90

5.4 Inhibitory effect of antibiotic combinations added to Mueller-Hinton agar against *C. coli* and *C. jejuni* 91

Table Page

5.5 Mean $\log_{10}$ CFU of *C. coli* strains UA40, UA44 and UA100 plated onto MH, M4 and M4 with blood 94

6.1 Differential characteristics of catalase positive *Campylobacter* species. 108

6.2 Biotyping scheme for *C. jejuni*, *C. coli*, and *C. laridis*. 110

6.3 Frequency of isolation of *Campylobacter* strains from colons of 40 cattle and 52 swine 116

6.4 The identification and classification of *Campylobacter* isolates from cattle and swine. 118

6.5 The G+C composition of total genomic DNA of isolates from cattle and swine 120

6.6 The plasmid profiles of isolates from swine determined by gel electrophoresis 130

8.1 The selective media used for quantitative comparisons of inhibitory effects of antibiotics on the growth of *C. coli* and *C. jejuni* 161

8.2 Range of MICs for 101 strains of *C. coli* and 60 strains of *C. jejuni* to eight antibiotics used in selective media for isolation of *Campylobacter* spp 163

8.3 The range of MICs of 14 *C. coli* strains to five antibiotics used in selective media for the isolation of *Campylobacter* spp. 165

8.4 The range of MICs of 8 *C. jejuni* strains to five antibiotics used in selective media for the isolation of *Campylobacter* spp. 166

8.5 Inhibitory effect of selective media on *C. coli* and *C. jejuni* 167

9.1 Strains of *Campylobacter* used in mating and hybridization experiments 179

9.2 The MIC and plasmid content of tetracycline-resistant isolates from the colon of cattle and swine 184

Table Page

9.3 The restriction fragment sizes of plasmids in *Campylobacter* strains UA466, $25a_1$, $25b_1$ and $29a_1$ 195

9.4 Southern transfer hybridization of plasmids from Tc^R and Tc^S *Campylobacter* isolates with pUA649 204

List of Figures

Figure Page

3.1 Scanning electron micrographs of *C. jejuni* at different magnifications 44

3.2 Scanning electron micrographs of *Campylobacter* cells 47

3.3 Trasmission electron micrograph of negative-stained cells of *C. jejuni* showing flagellation of both spiral and coccus-shaped cells 50

3.4 Transmission electron micrographs of thin sections of *C. jejuni* and *C. laridis* showing different morphological forms of the cells 52

6.1 Autoradiogram prepared using the slot blot hybridization of DNA from *Campylobacter* isolates from cattle and swine with ^{32}P-labelled DNA probe prepared from the total genomic DNA of *C. jejuni* UA580 122

6.2 Autoradiogram of the slot blot hybridization of DNA from *Campylobacter* isolates with ^{32}P-labelled DNA probe from *C. coli* UA578 125

6.3 The autoradiograms of DNA from unidentifiable isolates hybridized with DNA probes prepared from 51a$_3$, *C. laridis* UA577, *C. hyointestinalis* UA564, and *C. fetus* subsp. *fetus* UA60 127

7.1 Autoradiograms of dot blot hybridizations of *Campylobacter* strains with total genomic DNA from *C. jejuni* (UA580) prepared as a DNA probe 147

7.2 Autoradiograms of dot blot hybridizations with *C. coli* (UA578) using the total genomic DNA as probe 150

7.3 Autoradiograms of dot blot hybridizations with DNA probe prepared from sonicated total genomic DNA from *C. coli*(UA578) 152

9.1 The restriction map of *C. jejuni* Tc^R region of the plasmid pUA466 176

Figure Page

9.2 Comparison of the sensitivity and specificity of two DNA probes: the 1.8 kb *Hinc*II fragment from pUA466 and the 5 kb *tetM* probe from pJI3 186

9.3 Dot blot hybridization with 1.8 kb *Hinc*II fragment from pUA466 189

9.4 Southern blot of plasmids from *Campylobacter* isolates hybridized with the 1.8 kb *Hinc*II fragment from pUA466 192

9.5 The restriction fragment patterns of *Campylobacter* plasmids digested with *Acc*I and their Southern hybridization with the 1.8 kb *Hinc*II fragment probe 196

9.6 The restriction fragment pattern of *Campylobacter* plasmids digested with *Hinc*II and their Southern hybridization with the 1.8 kb *Hinc*II fragment probe 198

9.7 The Southern blot hybridization of the total genomic DNA from $25b_1$ with the 1.8 kb *Hinc*II probe 201

9.8 Southern blot hybridization of total plasmids from *Campylobacter* strains with pUA649 205

9.9 Hybridization of chromosomal DNA of *Campylobacter* digested with *Hinc*II to plasmid pUA649 207

9.10 Autoradiogram prepared from dot blot hybridization of *Campylobacter* strains with pUA649 210

Abbreviations

ATCC	American Type Culture Collection
CFU	colony forming units
CIP	Collection of Institute Pasteur
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
NCTC	National Collection of Type Cultures
no.	number
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
Tris	tris (hydroxymethyl) aminomethane
UV	ultraviolet

1. Introduction

Since the work of Butzler *et al.* (1973) and Skirrow (1977) the bacteria now referred to as *Campylobacter jejuni* and *Campylobacter coli* have been recognized as a major cause of human gastroenteritis. However, *C. coli* only accounts for 5% of the gastroenteritis caused by *Campylobacter* organisms, whereas *C. jejuni* accounts for the rest (Karmali and Skirrow, 1984).

The cells of the genus *Campylobacter* are small (0.5 to 8 µm long and 0.2 to 0.5 µm wide) relative to other enteric organisms, so that they can be selectively filtered through membranes with 0.65 µm pore size. They are nonsporeforming, gram-negative bacteria. Cells are spiral-shaped in young cultures but coccoid forms predominate in cultures over 72 h old (Smibert, 1985). The spiral-shaped cells have a characteristic darting motility when viewed by phase contrast microscopy. Usually a single polar flagellum is found at one or both ends of the cell (Smibert, 1978). Most members of this genus are microaerophilic, though an aerotolerant group has recently been described (Neill *et al.*, 1985).

Campylobacter spp. use amino acids and tricarboxylic acid cycle intermediates as their principal energy sources. Carbohydrates are neither fermented nor oxidized (Smibert, 1978). The guanine and cytosine (G+C) content of *Campylobacter* spp. ranges from 29 to 38 mol% (Smibert, 1978). The biochemical characteristics and the G+C content distinguish *Campylobacter* spp. from members of the genus *Vibrio*.

The taxonomy and nomenclature of *Campylobacter* spp. have undergone many changes, especially with respect to the nomenclature of *Campylobacter fetus*, *C. jejuni* and *C. coli*. The system currently in use is based on the scheme proposed by Véron and Chatelain (1973). Both the Smibert (1974) and Véron and Chatelain (1973) nomenclatures have been widely used, resulting in considerable confusion. Studies correlating differential characteristics and DNA homology of *C. coli* and *C. jejuni* showed that these organisms are from two distinct species, based on 53 to 96% homology within species and ≤ 49% homology between species (Hébert *et al.*, 1984; Owen and Leaper, 1981; Roop *et al.*, 1984; Ursing *et al.*, 1983). *C. coli* and *C. jejuni* are phenotypically similar and only hippurate hydrolysis can be used to differentiate them (Harvey, 1980; Leaper and Owen, 1981). The term "*C. jejuni/coli*" has been used by some investigators when these organisms have not been differentiated, therefore, it is not clear from many reports whether the organisms in question were *C. jejuni* or *C. coli* (Karmali and Fleming 1979; Jones *et al.*, 1980).

The overall objective of this study was to examine some of the factors that may contribute to the lower incidence of gastroenteritis caused by *C. coli* compared with *C. jejuni*, and to characterize fresh isolates of *C. coli* and *C. jejuni* obtained from animals. The hypotheses for the lower reported incidence of *C. coli* are as follows:

1. the present method of isolating *Campylobacter* strains may not be suitable for the isolation of *C. coli*; and
2. *C. coli* and *C. jejuni* cannot be differentiated reliably using phenotypic characteristics, as a result the significance of *C. coli* may be underestimated.

The experiments conducted to study these hypotheses included:

1. determination of the viability of different forms of *C. coli* and *C. jejuni* so that the physiological state of the organisms could be standardized for use in further studies;
2. evaluation of basal media used for culturing the organisms so that a suitable medium could be used for further study of the use of antibiotics as selective agents;
3. isolation of *C. coli* and *C. jejuni* from swine and cattle using the filtration technique and a selective medium so that the effects of antibiotics on fresh isolates and laboratory strains could be compared;
4. characterization of fresh isolates from swine and cattle using phenotypic characteristics and DNA (G+C content and homology) studies to determine the reliability of differentiating *C. coli* and *C. jejuni*;
5. characterization of fresh isolates of *C. coli* and *C.*

jejuni using biotyping, plasmid profiles and antibiotic susceptibilities; and

6. determination of the susceptibilities of laboratory strains and fresh isolates of *C. coli* and *C. jejuni* to antibiotics used in selective media.

During the determination of the antibiotic susceptibility patterns of fresh isolates of *C. coli* and *C. jejuni*, tetracycline-resistant strains were encountered. Some of these strains were from animals which had not been exposed to antibiotics. The mechanisms of tetracycline-resistance in these strains were further studied using DNA probes from *C. jejuni* and *Streptococcus agalactiae*.

1.1 Bibliography

Butzler, J. P., P. Dekeyser, M. Detrain, and F. Dehaen. 1973. Related vibrio in stools. J. Pediatr. 82:493-495.

Harvey, S. M. 1980. Hippurate hydrolysis by *Campylobacter fetus*. J. Clin. Microbiol. 11:435-437.

Hébert, G. A., P. Edmonds, and D. J. Brenner. 1984. DNA relatedness among strains of *Campylobacter jejuni* and *Campylobacter coli* with divergent serogroup and hippurate reactions. J. Clin. Microbiol. 20:138-140.

Jones, D. M., J. Eldridge, and B. Dale. 1980. Serological response to *Campylobacter jejuni/coli* infection. J. Clin. Pathol. 33:767-769.

Karmali, M. A., and P. C. Fleming. 1979. Campylobacter enteritis. Can. Med. Assoc. J. 23:1525-1532.

Karmali, M. A., and M. B. Skirrow. 1984. Taxonomy of the genus *Campylobacter*, p.1-20. *In* J. P. Butzler (ed.) Campylobacter infection in man and animals. CRC Press, Inc., Boca Raton, Fla.

Leaper, S., and R. J. Owen. 1981. Identification of catalase-producing *Campylobacter* species based on biochemical characteristics and on cellular fatty acid composition. Curr. Microbiol. 6:31-35.

Neill, S. D., J. N. Campbell, J. J. O'Brien, S. T. C. Weatherup, and W. A. Ellis. 1985. Taxonomic position of *Campylobacter cryaerophila* sp. nov. Int. J. Syst. Bacteriol. 35:342-356.

Owen R. J., and S. Leaper. 1981. Base composition, size and nucleotide sequence similarities of genome deoxribonucleic acids from species of the genus *Campylobacter*. FEMS Microbiol. Lett. 12:395-400.

Roop, R. M., II, R. M. Smibert, J. L. Johnson, and N. R. Krieg. 1984. Differential characteristics of catalase-positive campylobacters correlated with DNA homology groups. Can. J. Microbiol. 30:938-951.

Skirrow, M. B. 1977. Campylobacter enteritis: a "new" disease. Br. Med. J. 2:9-11.

Smibert, R. M., 1974. *Campylobacter*, p.207-212. *In* R. E. Buchanan, and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams and Wilkins Co., Baltimore.

Smibert, R. M. 1978. The genus *Campylobacter*. Annu. Rev. Microbiol. 32:673-709.

Smibert, R. M. 1985. *Campylobacter*, p.111-118. *In* N. R. Krieg (ed.), Bergey's manual of systematic bacteriology, vol 1. Williams and Wilkins Co., Baltimore.

Ursing, J., M. Walder, and K. Sandstedt. 1983. Base composition and sequence homology of deoxyribonucleic acid of thermotolerant *Campylobacter* from human and animal sources. Curr. Microbiol. 8:307-310.

Véron, M., and R. Chatelain, 1973. Taxonomic study of the genus *Campylobacter* Sebald and Véron and designation of the neotype strain for the type species, *Campylobacter fetus* (Smith and Taylor) Sebald and Véron. Int. J. Syst. Bacteriol. 23:122-134.

2. Review of Literature

2.1 Historical significance of *C. jejuni* and *C. coli*

Members of the genus *Campylobacter* were originally referred to as "microaerophilic vibrios" (McFadyean and Stockman, 1913), and were classified as *Vibrio fetus*, *V. jejuni* and *V. coli* (Smith and Taylor, 1919; Jones and Little, 1931; Doyle, 1944). The first documented outbreak of human gastrointestinal disease associated with microaerophilic vibrios occurred in 1946 in two penal institutions in Illinois (Levy, 1946). Of the 6,019 inmates, 357 contracted gastroenteritis which was attributed to "vibrio-like" organisms observed by microscopic examination of stool specimens. However, these organisms could not be characterized because they would not grow on laboratory media. King (1957) observed a group of organisms from blood cultures of patients with gastroenteritis which she designated as "related" vibrios. She noticed that these organisms were similar to *V. fetus*, except that they had a higher optimum growth temperature.

Dekeyser *et al.* (1972) developed a filtration technique for selective isolation of microaerophilic vibrios from stool specimens. This enabled Butzler *et al.* (1973) to screen stool specimens from 800 children and 100 adults with diarrhea. They isolated these organisms from the stools of 5.2% and 4.0% of each group, respectively. However, it was not until Skirrow (1977) developed a direct plating method that the importance of these organisms as a cause of gastroenteritis was fully recognized.

The recognition *Campylobacter* spp. as important causes of human gastroenteritis stimulated many research studies that have dramatically increased our knowledge of them and more species have been described. At present, the genus consists of ten species, of which five are listed in the current edition of *Bergey's Manual of Systematic Bacteriology* (Smibert, 1985) and the other five species have been recognized by the International Committee of Systematic Bacteriology (Anonymous, 1985a,b; Moore *et al.*, 1985; Neill *et al.*, 1985). The relationship between the current nomenclature proposed by Véron and Chatelain (1973) and other nomenclatures is shown in Table 2.1.

2.2 Incidence and geographical distribution of *Campylobacter* enteritis

Since the introduction of Skirrow's medium (1977), many outbreaks of human campylobacteriosis have been reported and reviews of these outbreaks have been published (Blaser and Reller, 1981; Blaser *et al.*, 1984; Doyle, 1981; Robinson and Jones, 1981; Speelman and Struelens, 1984). *Campylobacter* enteritis is an important diarrheal disease in both developed and developing countries. It ranks with or next to *Salmonella* spp. in importance (Blaser *et al.*, 1984; Finch and Riley, 1984). In developed countries, the highest incidence of *Campylobacter* enteritis is reported during the

Table 2.1 Changes in nomenclature of *C. fetus, C. jejuni* and *C. coli*

Present nomenclature (Véron and Chatelain, 1973)	Past nomenclature	Reference
C. fetus	*V. fetus*	Smith and Taylor, 1919
C. fetus subsp. *fetus*	*V. fetus* var. *intestinalis*	Florent, 1959
	C. fetus subsp. *intestinalis*	Smibert, 1974
C. fetus subsp. *venerealis*	*C. fetus* var. *venerealis*	Florent, 1959
	C. fetus subsp. *fetus*	Smibert, 1974
C. jejuni	*V. jejuni*	Jones *et al.*, 1931
	"Related vibrios"	King, 1957
	C. fetus subsp. *jejuni*	Smibert, 1974
C. coli	*V. coli*	Doyle, 1944
	"Related vibrios"	King, 1957
	C. fetus subsp. *jejuni*	Smibert, 1974

summer months. In Hong Kong, the peak season for outbreaks of *Campylobacter* enteritis occurs during the cool months of the year (Ho and Wong, 1985). In developing countries, there is a higher incidence of *Campylobacter* enteritis than in industrialized countries, and there is no apparent seasonal trend (Blaser and Reller, 1981). In a study conducted in Bangladesh, 17.7% of asymptomatic persons were shown to be carriers of *C. jejuni* (Blaser *et al.*, 1980a). Asymtomatic carriers have not been reported in developed countries.

Campylobacter enteritis can affect all age groups. However, it is suggested that the incidence is highest in young children (Karmali and Fleming, 1979). In developing countries, the majority of infections are found in children under the age of 5 years (Blaser *et al.*, 1981). The frequency of isolation of *C. coli* is much lower than that of *C. jejuni*. *C. coli* accounts for only 2 to 5% of outbreaks of *Campylobacter* enteritis in United Kingdom and Canada (Karmali *et al.*, 1983; Karmali and Skirrow, 1984; Thompson *et al.*, 1986). A survey conducted in Hong Kong indicated an exceptionally high rate of 41% of outbreaks of *Campylobacter* enteritis attributed to *C. coli* (Ho and Wong, 1985).

2.3 Vehicles of transmission and reservoirs of *Campylobacter*

Based on epidemiological studies, it appears that transmission of *C. jejuni* and *C. coli* occurs by consuming contaminated food and water or by contact with fecal material from infected animals or persons (Blaser and

Reller, 1981; Karmali and Fleming, 1979). Raw milk and water have often been implicated epidemiologically as the sources of organisms causing *Campylobacter* enteritis. However, *C. jejuni* and *C. coli* have seldom been isolated from suspected samples of milk and water (Robinson and Jones, 1981; Wright *et al.*, 1983).

C. jejuni is found in cattle, sheep, dogs and poultry while *C. coli* is mainly found in hogs (Blaser and Reller, 1981; Bokkenheuser and Mosenthal, 1981; Doyle, 1981; Grant *et al.*, 1980; Karmali and Skirrow,1984; Park *et al.*, 1981, 1983). Doyle (1981) reported that wild ducks commonly shed *C. jejuni* in their feces. Knill *et al.* (1978) assayed 84 samples of water for the presence of *C. jejuni* and found the organism present in 7 of 34 seawater samples and 37 of 50 fresh water samples. Subsequently, Knill *et al.* (1981) reported that *C. jejuni* was isolated from 53% of water samples collected from rivers. Blaser *et al.* (1980b) showed that *C. jejuni* survives in water at 4°C for 1 to 4.5 weeks and up to 4 days at 25°C.

2.4 Differential characteristics of *C. jejuni* and *C. coli*

C. jejuni and *C. coli* are catalase-positive and they can be differentiated from other catalase-positive species (*C. fetus*, *C. hyointestinalis*, *C. cinaedi*, *C. fennelliae* and *C. laridis*) by their growth at different temperatures, with optimum growth at 42°C but failure to grow at 25°C (King, 1957), susceptible to 40 µg/mL (30 µg disk) nalidixic acid and resistance to 64 μg/mL (30 μg disk) cephlothin (Karmali and Skirrow, 1984).

The differentiation of *C. jejuni* and *C. coli* is more difficult. The most reliable test is the hippurate hydrolysis test (Harvey, 1980; Kodaka *et al*., 1982). *C. jejuni* hydrolyzes hippurate to glycine and benzoic acid, but *C. coli* does not. However, hippurate-negative *C. jejuni* have recently been reported (Hébert *et al*., 1984; Roop *et al*., 1984; Totten *et al*., 1985b) and only DNA homology studies can be used to differentiate these strains from *C. coli*. In the taxonomic study of *Campylobacter* spp. conducted by Véron and Chatelain (1973), *C. coli* strains were tolerant to brilliant green (10 μg/mL), triphenyltetrazolium chloride (TTC) (1 mg/mL), and glucose (8%) but the *C. jejuni* strains were not. Subsequently, it was shown that these criteria are not reliable for the differentiation of *C. coli* and *C. jejuni* (Skirrow and Benjamin, 1980b; Karmali and Skirrow, 1984). Skirrow and Benjamin (1980b) used the ability to grow at different temperatures to differentiate *Campylobacter* spp. They showed that *C. coli* NCTC 11353 grew at 30.5°C but *C. jejuni* NCTC 11168 did not, but this test required controlled conditions and was not suitable for routine use (Skirrow and Benjamin, 1980a,b).

2.5 Epidemiological markers for *C. coli* and *C. jejuni*

Several epidemiological markers have been developed for strain differentiation of *C. coli* and *C. jejuni*. The first biotyping scheme for *Campylobacter* spp. was proposed by Skirrow and Benjamin (1980a). Using H_2S production in an iron medium (with 0.05% each of ferrous sulfate, sodium metabisulfite and sodium pyruvate), they were able to differentiate *C. jejuni* into two biotypes. Lior (1984) expanded this biotyping scheme to include the DNA hydrolysis test. He was able to differentiate *C. jejuni* into four biotypes and *C. coli* into two biotypes. For the biotyping of *C. jejuni*, Hébert *et al.* (1982) proposed a scheme which included DNA hydrolysis and growth on a charcoal-yeast agar. Roop *et al.* (1984) used alkaline phosphatase activity and DNA hydrolysis for the biotyping of *C. jejuni* and *C. coli*. Using this biotyping scheme, Roop *et al.* (1984) separated *C. jejuni* (20 strains) and *C. coli* (12 strains) into four biotypes for each species. The biotyping schemes proposed by Skirrow and Benjamin (1980a) and the Lior (1984) systems have been used in other epidemiological studies, for example, Kapperud *et al.* (1984) and Alabi *et al.* (1986). The usefulness of the system proposed by Roop *et al.* (1984) has not been further evaluated.

Two serotyping systems have been developed for differentiating *C. coli* and *C. jejuni* strains (Lior *et al.*, 1982; Penner and Hennessy, 1980). The Lior and Penner serotyping systems have been compared (Jones *et al.*, 1985;

Kaijser and Sjogren, 1985; Patton *et al.*, 1985). The results indicated that both systems were comparable in distinguishing isolates from human and non-human sources and that they were useful for epidemiological studies. However, greater discrimination was obtained when both methods were applied to the same organisms (Jones *et al.*, 1985).

Other epidemiological markers that have been proposed include a phage typing system (Grajewski *et al.*, 1985), plasmid profiles (Bopp *et al.*, 1985; Tenover *et al.*, 1984), chromosomal restriction patterns (Penner *et al.*, 1983), auxotyping (Tenover, *et al.*, 1985) and lectin interaction (Wong *et al.*, 1986). These markers have not been evaluated for use in epidemiological studies, but they may be used in combination with biotyping or serotyping schemes for strain differentiation.

2.6 Antibiotic resistance of *Campylobacter* organisms

Antibiograms of bacteria are determined for several reasons: generally (a) to identify an antibiotic for therapeutic use; but also (b) to differentiate bacterial strains for taxonomic or epidemiological purposes; and (c) for use as selective agents in laboratory media for the enrichment or selective growth of bacteria.

Different techniques have been used to determine the *in vitro* susceptibility patterns of *Campylobacter* spp. (Ahonkhai *et al.*, 1981; Gebhart *et al.*, 1985; Karmali *et al.*, 1980; Vanhoof *et al.*, 1980). The earlier classification schemes (Smibert, 1974) did not differentiate between *C. jejuni* and *C. coli*, therefore, data for antibiotic resistance of *Campylobacter* spp. must be interpreted with care.

(a) Antibiotics used for differentiation of *Campylobacter* spp.

In general, *C. jejuni* and *C. coli* are resistant to cephalothin and susceptible to nalidixic acid. The concentrations of cephalothin and nalidixic acid that differentiate *C. jejuni* and *C. coli* from *C. fetus* and *C. laridis* are 64 µg/mL (Karmali and Skirrow, 1984) and 40 µg/mL (Karmali and Skirrow, 1984), respectively. Alternatively, disks containing 30 µg cephalothin or nalidixic acid can be used (Karmali *et al.*, 1980; Morris and Patton, 1985).

In a study by Vanhoof *et al.* (1978), 3% of 95 strains of *C. jejuni* and *C. coli* were found to be resistant to nalidixic acid, with a minimal inhibition concentration (MIC) greater than 40µg/mL. In addition, spontaneous mutation of laboratory strains of *C. coli* and *C. jejuni* to nalidixic acid resistance has been reported (Taylor *et al.*, 1985). Cephalothin susceptible strains of *C. coli* and *C. jejuni* have also been reported (Karmali and Skirrow, 1984; Brooks *et al.*, 1986; Mégraud and Elharrif, 1985). Other antibiotics have also been evaluated for taxonomic use. Wang *et al.* (1984) showed that most strains of *C. coli* are resistant to clindamycin, erythromycin, rosaramicin and Sch32063, and that using concentrations of 2, 8, 2 and 2 µg/mL, respectively. They may be used to differentiate between *C. coli* and *C. jejuni*.

(b) Antibiotics used in selective media

Campylobacter organisms do not ferment or oxidize carbohydrates and their biochemical characteristics cannot be used for development of selective or differential media. As a result, antibiotics have been used as selective agents for the isolation of *Campylobacter* spp. The antibiotics most commonly used as selective agents include: cephalosporins, trimethoprim, vancomycin, rifampicin, polymyxins, bacitracin, novobiocin and fungicides.

1. **Cephalosporins.** Cephalosporins have been developed and improved to maintain activity against β-lactamase producing gram-negative bacteria such as *Enterobacter*, *Serratia*, *Proteus* and *Pseudomonas* spp. Therefore, the specific antibacterial spectrum differs between the cephalosporins (Goto, 1982; Turck, 1982; Webber and Yoshida, 1982). In general, *C. jejuni* and *C. coli* are resistant to cephalothin, cephaloridine, cefazolin and cefoperazone.
2. **Trimethoprim.** Trimethoprim has a broad spectrum of antibacterial activity. It is active against most of the *Enterobacteriaceae* (MIC <0.2 to 3 µg/mL), *Staphylococcus* spp. (MIC 0.2 to 1.6 µg/mL) and *Streptococcus* spp. (MIC

0.5 μg/mL) (Atkinson, 1980; Carlson *et al.*, 1983; Goossens *et al.*, 1985; Kucers and Bennett, 1975). However, *Clostridium* spp. and *Pseudomonas* spp. are resistant to trimethoprim, MICs >50 μg/mL (Kucers and Bennett, 1975). *C. jejuni* and *C. coli* are resistant to high levels of trimethoprim (Carlson *et al.*, 1983; Goossens *et al.*, 1985).

3. **Vancomycin.** Vancomycin is highly effective against the gram-positive cocci such as *Staphylococcus* spp. (MIC <0.1 to 6.3 μg/mL) and *Streptococcus* spp. (MIC 0.2 to 3.1 μg/mL) (Neu *et al.*, 1984). Gram-negative bacteria are generally resistant to vancomycin, for example the MIC of *E. coli* is >10μg/mL (Kucers and Bennett, 1975).
4. **Rifampicin.** Rifampicin is particularly active against *Staphylococcus* spp. (MIC <0.0016 to 12 μg/mL) and *Streptococcus* spp. (MIC 0.001 to 6.3 μg/mL) (Neu *et al.*, 1984). Gram-negative bacteria such as *E. coli* are inhibited by high concentrations of 10 to 100 μg/mL (Kucers and Bennett, 1975).
5. **Polymyxins.** All polymyxins have a similar antibacterial spectrum, but there are quantitative differences in their activity (Kucers and Bennett, 1975). *Proteus* spp. and *S. marcescens* and all gram-positive bacteria are resistant to polymyxin (Kucers and Bennett, 1975).
6. **Bacitracin.** Bacitracin is highly active against gram-positive bacteria, but not against gram-negative bacilli (Kucers and Bennett, 1975). The following ranges

have been reported as MICs for *C. perfringens*, *S. aureus* and *S. faecalis*: 0.002 to 0.25, 0.8 to 25 and 12.5 to >100 U/mL, respectively (Atkinson, 1980).

7. **Novobiocin.** Novobiocin is active against *Staphylococcus* spp., *C. perfringens* and other gram-positive bacilli, but *S. faecalis* is usually resistant (Kucers and Bennett, 1975).
8. **Antifungal agents.** Actidione (Whiffen, 1948) and amphotericin B (Kucers and Bennett, 1975) do not have antibacterial activity but inhibit fungal growth. Tetracycline and rifampicin, which are normally quite inactive against fungi, act synergistically with amphotericin B against some fungi (Kucers and Bennett, 1975).

2.7 Growth of *C. jejuni* and *C. coli* on laboratory media

C. jejuni and *C. coli* are microaerophilic, requiring a reduced oxygen (5-10%) environment for growth (Kiggins and Plastridge, 1956; Smibert, 1978). However, they possess a strictly respiratory form of metabolism (Hoffman *et al.*, 1979b). Studies have shown that their microaerophilic nature may be due to their sensitivity to exogenous superoxide anions and hydrogen peroxide, although they produce superoxide dismutase and catalase (Hoffman *et al.*, 1979a). Adding a mixture of ferrous sulfate, sodium metabisulfite and sodium pyruvate (FBP) to culture media enhances their aerotolerance without causing any physiological change in the organisms (George *et al.*, 1978; Hoffman *et al.*, 1979b). This is attributed to the ability of FBP to quench the superoxide anions and hydrogen peroxide present in culture media (Hoffman *et al.*, 1979b).

Campylobacter spp. can be cultured on a variety of basal media with various growth supplements (see Table 2.2). The most commonly used supplements are animal blood and FBP. *C. jejuni* grow well at 42°C (Janssen and Helstad, 1982). However, freeze-stressed *C. jejuni* require a lower initial incubation temperature (37°C) in a non-selective medium to allow subsequent growth at 42°C on a selective medium (Ray and Johnson, 1983). *C. jejuni* and *C. coli* can generally be stored by freezing at -70°C in the presence of glycerol (Goossens *et al.*, 1984). Heat-stressed cells are more sensitive to polymyxin B and colistin than unheated cells (Hanninen, 1982). Humphrey and Cruickshank (1985) demonstrated that *C. jejuni* are more sensitive to rifampicin and sodium deoxycholate after freezing or heating.

2.8 Isolation methods

Three main techniques have been developed for isolation of *C. jejuni* and *C. coli* from human and environmental samples, including, filtration through a 0.65 µm pore size membrane, growth in selective media or enrichment of samples suspected of containing *Campylobacter* organisms.

Table 2.2 Basal media and supplements for growth of *C. jejuni* and *C. coli*

Basal media	References
Brucella agar or broth	Blaser *et al.*,1979; Border *et al.*, 1974; Hoffman *et al.*, 1979a, b; Trueblood and Tucker, 1957
Mueller Hinton agar or broth	Barot and Bokkenheuser, 1984; Lior *et al.*, 1982; Logan and Trust, 1984
Thioglycollate medium	Butzler and Skirrow, 1979
Columbia blood agar	Bolton and Robertson, 1982; Goossens *et al.*, 1983
Blood agar base no.2	Butzler and Skirrow, 1979; Skirrow, 1977
Nutrient broth no.2 with agar	Bolton and Robertson, 1982; Bolton and Coates, 1983
Chemically defined media	Tenover *et al.*, 1985
Special formulations	Mehlman and Romero, 1982; Rosef, 1981
Supplements	
FBP[1]	George *et al.*, 1978
Charcoal	Bolton and Coates, 1983; Lander and Gill, 1980
Sheep or horse blood	Border *et al.*, 1974; Lander and Gill, 1980
Hematin	Border *et al.*, 1974; Wesley *et al.*, 1983
Isovitalex[2]	Rosef *et al.*, 1983

[1] FBP: a mixture of ferrous sulphate, sodium metabisulfite, and sodium pyruvate

[2] Isovitalex™: a supplement for fastidious organisms (BBL, Becton, Dickinson and Co., MD, U.S.A.)

2.8.1 Filtration

The filtration method is based on the fact that *Campylobacter* cells are smaller in diameter than most other enteric bacteria. Using a 0.65 µm pore size membrane, *C. jejuni* and *C. coli* can be physically separated from the background flora of fecal samples (Dekeyser *et al*., 1972). The filtrate can then be cultured on solid media. This method is cumbersome and it became obsolete with the introduction of selective growth media. A modified filtration method was developed by Steele and McDermott (1984). A suspension of the sample or specimen is spotted onto a 45 µm pore size membrane placed on the surface of non-selective agar plates. During an initial pre-incubation period (about one hour), *Campylobacter* organisms migrate through the pores of the membrane onto the agar surface. The membrane is then removed from the agar surface and the plates are incubated. Steele and McDermott (1984) used this modified filtration method to examine 1,000 stool specimens. They obtained a higher recovery of *Campylobacter* organisms with this method than using a selective medium containing vancomycin, trimethoprim and colistin.

Mégraud and Elharrif (1985) showed that a membrane of 0.65 µm pore size is more efficient than the 0.45 µm pore size. Using the 0.65 µm pore size membrane, they reported fewer contaminants than using the classical pressure filtration technique. They also compared the modified filtration technique with selective antibiotic-containing media. They detected *Campylobacter* in 72 of 1,600 stool specimens. Only 59 *Campylobacter* strains were isolated by the filtration method, of which five *C. jejuni* and eight *Campylobacter*-like organisms (CLO) isolates did not grow on selective media. Six of these 13 strains were sensitive to colistin or cephalothin.

2.8.2 Selective media

As early as 1972, Dekeyser *et al*. in Butzler's laboratory used a medium containing antibiotics in combination with the filtration method, for the isolation of *Campylobacter* organisms. To eliminate the filtration step, Skirrow (1977) developed a medium using a combination of antibiotics for primary isolation of *Campylobacter* organisms (see Table 2.3). Since then, many other selective media have been described in which different combinations of antibiotics are proposed as selective agents (see Table 2.3).

The concentration of antibiotics in Skirrow's medium was formulated for use with blood agar base number 2 (Oxoid). If a different basal medium is used, it may be necessary to change the concentration of antibiotics used in the medium (Butzler and Skirrow, 1979). This observation is supported by Karmali and Fleming (1979), who used Columbia blood agar base (Gibco, Burlington, Ontario) as the basal medium. They found it necessary to raise the concentration of polymyxin B from 2,500 to 5,000 IU/L of medium. Lysed

Table 2.3 Combinations of antibiotics used in media for selective isolation of *C. coli* and *C. jejuni*.

Medium reference	Antibiotics (per L of medium)	
Dekeyser *et al.*, 1972	bacitracin	(25,000 IU)
	polymyxin B	(10,000 IU)
	novobiocin	(5 mg)
	actidione	(50 mg)
Skirrow, 1977	trimethoprim	(5 mg)
	polymyxin B	(2,500 IU)
	vancomycin	(10 mg)
Lauwers *et al.*, 1978 "Butzler's medium, Oxoid"	bacitracin	(25,000 IU)
	novobiocin	(5 mg)
	actidione	(50 mg)
	colistin	(10,000 IU)
	cephalothin	(15 mg)
Goossens *et al.*, 1983 "Butzler's medium Virion"	rifampicin	(10 mg)
	cefoperazone	(15 mg)
	colistin	(10,000 IU)
	amphotericin B	(2 mg)
Blaser *et al.*, 1979 "Campy-BAP"	cephalothin	(15 mg)
	trimethoprim	(5 mg)
	vancomycin	(10 mg)
	polymyxin B	(2,500 IU)
	amphotericin B	(2 mg)
Bolton and Robertson, 1982 "Preston medium"	polymyxin B	(5,000 IU)
	rifampicin	(10 mg)
	trimethoprim	(10 mg)
	actidione	(100 mg)
Bolton et al, 1984 "CCD agar"	cefazolin	(10 mg)
Rosef *et al.*, 1983	colistin	(10,000 IU)
	amphotericin B	(1 mg)
	cephalothin	(15 mg)
Wesley, *et al.*, 1983	polymyxin B	(20,000 IU)
	rifampicin	(25 mg)
	cefsulodin	(6.25 mg)
Waterman *et al.*, 1984	polymyxin B	(2,500 IU)
	trimethoprim	(5 mg)
	rifampicin	(10 mg)
	actidione	(100 mg)

horse blood is added to Skirrow's medium to neutralize trimethoprim antagonists, such as thymidine (Butzler and Skirrow, 1979). Bopp *et al.* (1982) suggested that sheep blood can be used instead of lysed horse blood without affecting the selectivity of Skirrow's medium.

After discontinuing the use of the filtration technique, Butzler and co-workers modified the medium used by Dekeyser *et al.* (1972) by adding cephalothin and colistin in place of polymyxin B (Lauwers *et al.*, 1978). The Skirrow (1977) and Lauwers *et al.* (1978) media for primary isolation of *Campylobacter* from stool samples were evaluated by the Centers for Disease Control, Atlanta (Patton *et al.*, 1981). No difference in isolation frequency was observed between media, although the Lauwers *et al.* (1978) medium was more effective in reducing *Proteus* contaminants. However, Patton *et al.* (1981) showed that by increasing the concentration of colistin from 10,000 to 40,000 IU/L, a higher isolation rate of *Campylobacter* organisms was achieved, because of the greater inhibition of coliform bacteria.

The formulation proposed by Lauwers *et al.* is usually referred to as "Butzler's medium". It is available commercially (Oxoid Ltd., Basingstoke, England), but cefazolin is used in place of cephalothin, and the basal medium is Columbia blood agar base in place of thioglycollate medium (Butzler and Skirrow, 1979). Butzler's medium was subsequently modified by Goossens *et al.* (1983). It is referred to as "Butzler's medium Virion" to distinguish it from the original "Butzler medium". The antibiotic combination used in "Butzler's medium Virion" is shown in Table 2.3. This medium suppresses the fecal flora, especially *Pseudomonas aeruginosa* and *Enterobacteriaceae*. Both of the "Butzler's media" can be incubated at 37°C, without overgrowth by the fecal microflora. Use of the lower incubation temperature allows isolation of *C. fetus*. This could eliminate the need for additional incubation facilities at 42°C. Blaser *et al.* (1979) proposed a modification of Skirrow's medium in which cepholothin and amphotericin B were included in addition to trimethoprim, polymyxin B and vancomycin. This medium is known as Campy-BAP.

Researchers at the Public Health Laboratory, Preston, England found Skirrow's media to be suitable for isolation of *Campylobacter* organisms from stool specimens, but less suitable for isolation of these organisms from animal and environmental samples. They developed a medium known as Preston medium (Bolton and Robertson, 1982), for isolation of *Campylobacter* organisms from "any kind of specimen". Subsequently, Bolton and Coates (1983) found that a combination of charcoal, ferrous sulfate and sodium pyruvate was as effective as blood in supporting the growth of *Campylobacter* spp. Thus, a blood-free selective medium (charcoal-cefazolin-sodium deoxycholate or CCD agar) was developed (Bolton *et al.*, 1984). Subsequently cefazolin was replaced by cefoperazone (Hutchinson and Bolton, 1984). This medium is similar to that described by Karmali *et al.*, (1986), except that Karmali *et al.* (1986) included vancomycin and actidione in addition to cefoperazone as selective agents.

Several media have been developed to recover *Campylobacter* organisms from foods (Table 2.3). Most of these media are designed for use in combination with an enrichment medium. Rosef *et al.* (1983) used only amphotericin B, colistin and cephalothin in a basal medium that is used for culturing gonococci. Wesley *et al.* (1983) used a combination of polymyxin, rifampicin and cefsulodin for the isolation of *Campylobacter* organisms. The concentrations of polymyxin and rifampicin are higher than those used in other solid media. Waterman *et al.* (1984) used a medium with the same antibiotics as Preston medium, but the concentrations of polymyxin and trimethoprim are lower.

2.8.3 Enrichment media

Fluid enrichment media have been developed which have greatly increased the sensitivity of media for detection of *Campylobacter* organisms in milk, water and other samples in which there may only be a few cells (Barot and Bokkenheuser, 1984; Doyle and Roman, 1982; Lovett *et al.*, 1983; Martin *et al.*, 1983; Park *et al.*, 1983; Rogel *et al.*, 1985; Tanner and Bullin, 1977; Wesley *et al.*, 1983). The selective agents used are the same as those used in solid selective media. In stool samples, *Campylobacter* organisms are usually present in large numbers and enrichment is generally not necessary (Chan, *et al.*, 1985; Doyle, 1981; Hutchinson, and Bolton, 1983). However, if the stool specimens are transported, enrichment may increase the isolation rate (Chan *et al.*, 1985; Hodge and Terro, 1984)

2.9 Concluding remarks

The pioneer work of Butzler *et al.* (1973) and Skirrow (1977) initiated the interest in the genus *Campylobacter* that led to the recognition of *C. jejuni* as a major cause of human gastroenteritis. In this chapter, only the historical significance, epidemiology, taxonomy and classification, isolation and identification of *C. coli* and *C. jejuni* have been reviewed. Many other aspects such as the pathogenesis of *Campylobacter* enteritis (Walker *et al.*, 1986), survival and growth characteristics in foods (Doyle, 1984) have been reported, but these topics are not discussed because they are not directly related to this thesis. Several new species of *Campylobacter* have emerged in recent years, for example, *C. laridis* (Benjamin *et al.*, 1983), *C. cinaedi* (Fennell *et al.*, 1984; Totten *et al.*, 1985a), *C. fennelliae* (Fennell *et al.*, 1984; Totten *et al.*, 1985a), *C. hyointestinalis* (Gebhart *et al.*, 1985), and *C. pyloridis* (Marshall *et al.*, 1984). Some of these species have been shown to be of clinical importance although they do not play a major role in gastroenteritis.

2.10 Bibliography

Alabi, S. A., A. O. Coker, O. Dosunmu-Ogunbi, and T. Odugbemi. 1986. Biotype and serogroup distribution of *Campylobacter* isolates from children in Nigeria. J. Clin. Microbiol. 24:856-858.

Ahonkhai, V. I., C. E. Cherubin, M. F. Sierra, V. D. Bokkenheuser, M. A. Shulman, and A. C. Morsenthal. 1981. In vitro susceptibility of *Campylobacter fetus* subsp. *jejuni* to *N*-formimidoyl thienamycin, rosaramicin, cefoperazone, and other antimicrobial agents. Antimicrob. Agents Chemother. 20:850-851.

Anonymous. 1985a. Validation of the publication of new names and new combinations previously effectively published outside the IJSB, list no. 17. Int. J. Syst. Bacteriol. 35:223-225.

Anonymous. 1985b. Validation of the publication of new names and new combinations previously effectively published outside the IJSB, list no. 19. Int. J. Syst. Bacteriol. 35:535.

Atkinson, B. A. 1980. Species incidence, trends of susceptibility to antibiotics in the United States, and minimum inhibitory concentration, p. 607-722. *In* V. Lorian (ed.), Antibiotics in laboratory medicine. Williams and Wilkins, Baltimore.

Barot, M. S., and V. D. Bokkenheuser. 1984. Systematic investigation of enrichment media for wild-type *Campylobacter jejuni* strains. J. Clin. Microbiol. 20:77-80.

Benjamin, J., S. Leaper, R. J. Owen, and M. B. Skirrow. 1983. Description of *Campylobacter laridis*, a new species comprising the nalidixic acid resistant thermophilic campylobacter (NARTC) group. Curr. Microbiol. 8:231-238.

Blaser, M. J., I. D. Berkowitz, F. M. LaForce, J. Cravens, L. B. Reller, and W.-L. L. Wang. 1979. Campylobacter enteritis: Clinical and epidemiologic features. Ann. Intern. Med. 91:179-185.

Blaser, M. J., R. I. Glass, M. I. Huq, B. Stoll, G. M. Kibriya, and A. R. M. A. Alim. 1980a. Isolation of *Campylobacter fetus* subsp. *jejuni* from Bangladeshi children. J. Clin. Microbiol. 12:744-747.

Blaser, M. J., H. L. Hardesty, B. Powers, and W.-L. L. Wang. 1980b. Survival of *Campylobacter fetus* subsp. *jejuni* in biological milieus. J. Clin. Microbiol. 11:309-313.

Blaser, M. J., and L. B. Reller. 1981. Campylobacter enteritis. New Engl. J. Med. 305:1444-1452.

Blaser, M. J., D. N. Taylor, and R. A. Feldman. 1984. Epidemiology of *Campylobacter* infections, p. 143-161. *In*, J. P. Butzler (ed.), Campylobacter infection in man and animals. CRC Press, Boca Raton, Fla.

Bokkenheuser, V. D., and A. C. Mosenthal. 1981. Campylobacteriosis: A foodborne disease. J. Food Safety 3:127-143.

Bolton, F. J. and D. Coates. 1983. Development of a blood-free *Campylobacter* medium: Screening tests on basal media and supplements, and the ability of selected supplements to facilitate aerotolerance. J. Appl. Bacteriol. 54:115-125.

Bolton, F. J., D. N. Hutchinson, and D. Coates. 1984. Blood-free selective medium for isolation of *Campylobacter jejuni* from feces. J. Clin. Microbiol. 19:169-171.

Bolton, F. J., and L. Robertson. 1982. A selective medium for isolating *Campylobacter jejuni/coli*. J. Clin. Pathol. 35:462-467.

Bopp, C. A., K. A. Birkness, I. K. Wachsmuth, and T. J. Barrett. 1985. In vitro antimicrobial susceptibility, plasmid analysis, and serotyping of epidemic-associated *Campylobacter jejuni*. J. Clin. Microbiol. 21:4-7.

Bopp, C. A., J. G. Wells, and T. J. Barrett. 1982. Trimethoprim activity in media selective for *Campylobacter jejuni*. J. Clin. Microbiol. 16:808-812.

Border, M. M., B. D. Firehammer, and L. L. Myers. 1974. Tube culture method for viable counts of *Campylobacter fetus* (*Vibrio fetus*). Appl. Microbiol. 28:730-732.

Brooks, B. W., M. M. Garcia, A. D. E. Fraser, H. Lior, R. B. Stewart, and A. M. Lammerding. 1986. Isolation and characterization of cephalothin-susceptible *Campylobacter coli* from slaughter cattle. J. Clin. Microbiol. 24:591-595.

Butzler, J. P., P. Dekeyser, M. Detrain, and F. Dehaen. 1973. Related vibrio in stools. J. Pediatr. 82:493-495.

Butzler, J. P., and M. B. Skirrow. 1979. Campylobacter enteritis. Clin. Gastroenterol. 8:737-765.

Chan, R., B. Hannan, and R. Munro. 1985. Use of a selective enrichment broth for isolation of *Campylobacter* species from human feces. Pathol. 17:640-641.

Carlson, J. R., S. A. Thornton, H. L. DuPont, A. H. West, and J. J. Mathewson. 1983. Comparative in vitro activities of ten antimicrobial agents against bacterial enteropathogens. Antimicrob. Agents Chemother. 24:509-513.

Dekeyser, P., M. Gossuin-Detrain, J. P. Butzler, and J. Sternon. 1972. Acute enteritis due to related vibrio: First positive stool cultures. J. Infect. Dis. 125:390-392.

Doyle, L. P. 1944. A vibrio associated with swine dysentery. Am. J. Vet. Res. 5:3-5.

Doyle, M. P. 1981. *Campylobacter fetus* subsp. *jejuni*: An old pathogen of new concern. J. Food Prot. 44:480-488.

Doyle, M. P. 1984. *Campylobacter* in foods, p. 163-180. *In* J. P. Butzler (ed.), Campylobacter infection in man and animals. CRC Press, Inc., Boca Raton, Fla.

Doyle, M. P., and D. J. Roman. 1982. Recovery of *Campylobacter jejuni* and *Campylobacter coli* from inoculated foods by selective enrichment. Appl. Environ. Microbiol. 43:1343-1353.

Fennell, C. L., P. A. Totten, T. C. Quinn, D. L. Patton, K. K. Holmes, and W. E. Stamm. 1984. Characterization of *Campylobacter*-like organisms isolated from homosexual men. J. Infect. Dis. 149:58-66.

Finch, M. J., and L. W. Riley. 1984. *Campylobacter* infections in the United States: Results of an 11-State surveillance. Arch. Intern. Med. 144:1610-1612.

Florent, A. 1959. Les deux vibrioses génitales: La vibriose due a *V. fetus venerealis* et la vibriose d'origine intestinale due a *V. fetus intestinalis*, Meded. Veeartsenijsch. Rijksuniv. Gent. 3:1-60.

Gebhart, C. J., G. E. Ward, and H. J. Kurtz. 1985. In vitro activities of 47 antimicrobial agents against three *Campylobacter* spp. from pigs. Antimicrob. Agents Chemother. 27:55-59.

Gebhart, C. J., P. Edmonds, G. E. Ward, H. J. Kurtz, and D. J. Brenner. 1985. "*Campylobacter hyointestinalis*" sp. nov.: A new species of *Campylobacter* found in the intestines of pigs and other animals. J. Clin. Microbiol. 21:715-720.

George, H. A., P. A. Hoffman, R. M. Smibert, and N. R. Krieg. 1978. Improved media for growth and aerotolerance of *Campylobacter fetus*. J. Clin. Microbiol. 8:36-41.

Goossens, H., M. De Boeck, and J. P. Butzler. 1983. A new selective medium for the isolation of *Campylobacter jejuni* from human faeces. Eur. J. Clin. Microbiol. 2:389-394.

Goossens, H., M. De Boeck, H. Van Landuyt, and J. P. Butzler. 1984. Isolation of *Campylobacter jejuni* from human feces, p. 39-50. *In* J. P. Butzler (ed.), Campylobacter infection in man and animals. CRC Press, Inc. Boca Raton, Fla.

Goossens, H., P. De Mol, H. Coignan, J. Levy, O. Grados, G. Ghysels, H. Innocent, and J.-P. Butzler. 1985. Comparative in vitro activities of aztreonam, ciprofloxacin, norfloxacin, ofloxacin, HR 810 (a new cephalosporin), RU28965 (a new macrolide), and other agents against enteropathogens. Antimicrob. Agents Chemother. 27:338-392.

Goto, S. 1982. In vitro and in vivo antibacterial activity of moxalactam, an Oxa-β-lactam antibiotic. Rev. Infect. Dis. 4:S501-S510.

Grajewski, B. A., J. W. Kusek, and H. M. Gelfand. 1985. Development of a bacteriophage typing system for *Campylobacter jejuni* and *Campylobacter coli*. J. Clin. Microbiol. 22:13-18.

Grant, I. H., N. J. Richardson, and V. D. Bokkenheuser. 1980. Broiler chickens as potential source of *Campylobacter* infections in humans. J. Clin. Microbiol. 11:508-510.

Hanninen, M.-L. 1982. Effect of recovery medium on the isolation of *Campylobacter jejuni* before and after heat treatment. Acta. Vet. Scand. 23:416-424.

Harvey, S. M. 1980. Hippurate hydrolysis by *Campylobacter fetus* . J. Clin. Microbiol. 11:435-437.

Hébert, G. A., P. Edmonds, and D. J. Brenner. 1984. DNA relatedness among strains of *Campylobacter jejuni* and *Campylobacter coli* with divergent serogroup and hippurate reactions. J. Clin. Microbiol. 20:138-140.

Hébert, G. A., D. G. Hollis, R. E. Weaver, M. A. Lambert, M. J. Blaser, and C. W. Moss. 1982. 30 years of campylobacters: Biochemical characteristics and a biotyping proposal for *Campylobacter jejuni*. J. Clin. Microbiol. 15:1065-1073.

Ho, B. S. W., and W. T. Wong. 1985. A one-year survey of campylobacter enteritis and other forms of bacterial diarrhoea in Hong Kong. J. Hyg. (*Camb.*) 94:55-60.

Hoffman, P. S., H. A. George, N. R. Krieg, and R. M. Smibert. 1979a. Studies of the microaerophilic nature of *Campylobacter fetus* subsp. *jejuni*. II. Role of exogenous superoxide anions and hydrogen peroxide. Can. J. Microbiol. 25:8-16.

Hoffman, P. S., N. R. Krieg, and R. M. Smibert. 1979b. Studies of the microaerophilic nature of *Campylobacter fetus* subsp. *jejuni*. I. Physiological aspects of enhanced aerotolerance. Can. J. Microbiol. 25:1-7.

Hodge, D. S., and R. Terro. 1984. Comparative efficacy of liquid enrichment medium for isolation of *Campylobacter jejuni*. J. Clin. Microbiol. 19:434.

Humphrey, T. J., and J. G. Cruickshank. 1985. Antibiotic and deoxycholate resistance in *Campylobacter jejuni* following freezing or heating. J. Appl. Bacteriol. 59:65-71.

Hutchinson, D. N., and F. J. Bolton. 1983. Is enrichment culture necessary for the isolation of *Campylobacter jejuni* from faeces? J. Clin. Pathol. 36:1350-1352.

Hutchinson, D. N., and F. J. Bolton. 1984. Improved blood free selective medium for the isolation of *Campylobacter jejuni* from faecal specimens. J. Clin. Pathol. 37:956-957.

Janssen, D., and A. G. Helstad, 1982. Isolation of *Campylobacter fetus* subsp. *jejuni* from human fecal specimens by incubation at 35 and 42°C. J. Clin. Microbiol. 16:398-399.

Jones, F. S., and R. B. Little. 1931. Vibrionic enteritis in calves. J. Exp. Med. 53:845-851.

Jones, F. S., M. Orcutt, and R. B. Little. 1931. Vibrios

(*Vibrio jejuni*, n. sp.) associated with intestinal disorders of cows and calves. J. Exp. Med. 53:853-864.

Jones, D. M., E. M. Sutcliffe, and J. D. Abbott. 1985. Serotyping of *Campylobacter* species by combined use of two methods. Eur. J. Clin. Microbiol. 4:562-565.

Kaijser, B., and E. Sjogren. 1985. *Campylobacter* strains in Sweden. Acta Pathol. Microbiol. Immunol. Scand. Sect. B. 93:315-322.

Kapperud, G., J. Lassen, S. Lauwers, and O. Rosef. 1984. Serotyping and biotyping of *Campylobacter jejuni* and *Campylobacter coli* from sporadic cases and outbreaks in Norway. J. Clin. Microbiol. 19:157-160.

Karmali, M. A., S. DeGrandis, and P. C. Fleming. 1980. Antimicrobial susceptibility of *Campylobacter jejuni* and *Campylobacter fetus* subsp. *fetus* to eight cephalosporins with special reference to species differentiation. Antimicrob. Agents Chemother. 18:948-951.

Karmali, M. A., and P. C. Fleming. 1979. *Campylobacter* enteritis in children. J. Pediatr. 94:527-533.

Karmali, M. A., J. L. Penner, and P. C. Fleming, A. Williams, and J. N. Hennessy. 1983. The serotype and biotype distribution of clinical isolates of *Campylobacter jejuni* and *Campylobacter coli* over a three-year period. J. Infect. Dis. 147:243-246.

Karmali, M. A., A. E. Simor, M. Roscoe, P. C. Fleming. S. S. Smith, and J. Lane. 1986. Evaluation of a blood-free, charcoal-based, selective medium for the isolation of *Campylobacter* organisms from feces. J. Clin. Microbiol. 23:456-459.

Karmali, M. A., and M. B. Skirrow. 1984. Taxonomy of the genus *Campylobacter*, p. 1-20. *In* J. P. Butzler (ed.), Campylobacter infection in man and animals. CRC Press, Inc., Boca Raton, Fla.

Kiggins, E. M., and W. N. Plastridge. 1956. Effect of gaseous environment on growth and catalase content of *Vibrio fetus* cultures of bovine origin. J. Bacteriol. 72:397-400.

King, E. O. 1957. Human infections with *Vibrio fetus* and a closely related vibrio. J. Infect. Dis. 101:119-128.

Knill, M., W. G. Suckling, and A. D. Pearson. 1978.

Environmental isolation of heat-tolerant *Campylobacter* in the Southampton area. Lancet ii:1002-1003.

Knill, M., W. G. Suckling and A. D. Pearson. 1981. Campylobacters from water, p. 281-282. *In* D. G. Newell (ed.) Campylobacter: Epidemiology, pathogenesis and biochemistry. MTP Press Ltd., Lancaster.

Kodaka, H., G. L. Lombard, and V. R. Dowell, Jr. 1982. Gas-liquid chromatography technique for detection of hippurate hydrolysis and conversion of fumarate to succinate by microorganisms. J. Clin. Microbiol. 16:962-964.

Kucers, A., and M. McK. Bennett. 1975. The use of antibiotics, 2nd ed. Williams Heinemann Medical Books Ltd., London.

Lander, K. P., and K. P. W. Gill. 1980. Experimental infection of the bovine udder with *Campylobacter coli/jejuni*. J. Hyg. (*Camb.*) 84:421-428.

Lauwers, S., M. De Boeck, and J. P. Butzler. 1978. Campylobacter enteritis in Brussels. Lancet i:604-605.

Lauwers, S., L. Vlaes, and J. P. Butzler. 1981. *Campylobacter* serotyping and epidemiology. Lancet i:158-159.

Levy, A. J. 1946. A gastroenteritis outbreak probably due to a bovine strain of vibrio. Yale J. Biol. Med. 18:243-259.

Lior, H., 1984. New, extended biotyping scheme for *Campylobacter jejuni*, *Campylobacter coli*, and "*Campylobacter laridis*." J. Clin. Microbiol. 20:636-640.

Lior, H., D. L. Woodward, J. A. Edgar, L. J. Laroche, and P. Gill. 1982. Serotyping of *Campylobacter jejuni* by slide agglutination based on heat-labile antigenic factors. J. Clin. Microbiol. 15:761-768.

Logan, S. M., and T. J. Trust. 1984. Structural and antigenic heterogeneity of lipopolysaccharides of *Campylobacter jejuni* and *Campylobacter coli*. Infect. Immun. 45:210-216.

Lovett, J., D. W. Francis, and J. M. Hunt. 1983. Isolation of *Campylobacter jejuni* from raw milk. Appl. Environ.

Microbiol. 46:459-462.

Martin, W. T., C. M. Patton, G. K. Morris, M. E. Potter, and N. D. Puhr. 1983. Selective enrichment broth medium for isolation of *Campylobacter jejuni*. J. Clin. Microbiol. 17:853-855.

Marshall, B. J., H. Royce, D. I. Annear, C. S. Goodwin, J. W. Pearman, J. R. Warren, and J. A. Armstrong. 1984. Original isolation of *Campylobacter pyloridis* from human gastric mucosa. Microbios Lett. 25:83-88.

McFadyean, J., and S. Stockman. 1913. Report of the Departmental Committee appointed by the Board of Agriculture and Fisheries to enquire into epizootic abortion. Part III. Her Majesty's Stationary Office, London.

Mégraud, F., and Z. Elharrif. 1985. Isolation of *Campylobacter* species by filtration. Eur. J. Clin. Microbiol. 4:437-438.

Mehlman, I. J., and A. Romero. 1982. Improved growth medium for *Campylobacter* species. Appl. Environ. Microbiol. 43:615-618.

Moore, W. E. C., E. P. Cato, and V. H. Moore. 1985. Index of the bacterial and yeast nomenclatural changes published in the *International Journal of Systematic Bacteriology* since the 1980 approved lists of bacterial names (1 January 1980 to 1 January 1985). Int. J. Syst. Bacteriol. 35:382-407.

Morris, G. K., and C. M. Patton. 1985. *Campylobacter*, p. 302-308. *In* E. H. Lennette, A. Balows, W. J. Hausler, H. J. Shadomy (ed.), Manual of Clinical Microbiology, 4th ed. American Society for Microbiology, Washington, D.C.

Neill, S. D., J. N. Campbell, J. J. O'Brien, S. T. C. Weatherup, and W. A. Ellis. 1985. Taxonomic position of *Campylobacter cryaerophila* sp. nov. Int. J. Syst. Bacteriol. 35:342-356.

Neu, H. C., N.-X. Chin, and P. Labthavikul. 1984. Antibacterial activity of coumermycin alone and in combination with other antibiotics. Antimicrob. Agents Chemother. 2:687-689.

Park, C. E., Z. K. Stankiewicz, J Lovett, J. Hunt, and D. W. Francis. 1983. Effect of temperature, duration of incubation, and pH of enrichment culture on the recovery of *Campylobacter jejuni* from eviscerated market chickens. Can. J. Microbiol. 29:803-806.

Park, C. E., Z. K. Stankiewicz, J. Lovett, and J. Hunt. 1981. Incidence of *Campylobacter jejuni* in fresh eviscerated whole market chickens. Can. J. Microbiol. 27:841-842.

Patton, C. M., T. J. Barrett, and G. K. Morris. 1985. Comparison of the Penner and Lior methods for serotyping *Campylobacter* spp. J. Clin. Microbiol. 22:558-565.

Patton, C. M., S. W. Mitchell, M. E. Potter, and A. F. Kaufmann. 1981. Comparison of selective media for primary isolation of *Campylobacter fetus* subsp. *jejuni*. J. Clin. Microbiol. 13:326-330.

Penner, J. L., and J. N. Hennessy. 1980. Passive hemagglutination technique for serotyping *Campylobacter fetus* subsp. *jejuni* on the basis of soluble heat-stable antigens. J. Clin. Microbiol. 12:732-737.

Penner, J. L., J. N. Hennessy, S. D. Mills, and W. C. Bradbury. 1983. Application of serotyping and chromosomal restriction endonuclease digest analysis in investigating a laboratory-acquired case of *Campylobacter jejuni* enteritis. J. Clin. Microbiol. 18:1427-1428.

Ray, B., and C. Johnson. 1983. Growth requirements of freeze-stressed *Campylobacter jejuni*. Abstr. Annu. Meet. Am. Soc. Microbiol., New Orleans, p. 257.

Robinson, D. A., and D. M. Jones. 1981. Milk-borne *Campylobacter* infection. Br. Med. J. 282:1374-1376.

Roop, R. M., II, R. M. Smibert, and N. R. Krieg. 1984. Improved biotyping schemes for *Campylobacter jejuni* and *Campylobacter coli*. J. Clin. Microbiol. 20:990-992.

Rogel, M., B. Shpak, D. Rothman, and I. Sechter. 1985. Enrichment medium for isolation of *Campylobacter jejuni-Campylobacter coli*. Appl. Environ. Microbiol. 50:125-126.

Rosef, O. 1981. Isolation of *Campylobacter fetus* subsp. *jejuni* from the gallbladder of normal slaughter pigs, using an enrichment procedure. Acta Vet. Scand. 22:149-151.

Rosef, O., B. Gondrosen, G. Kapperud, and B. Underdal. 1983.

Isolation and characterization of *Campylobacter jejuni* and *Campylobacter coli* from domestic and wild mammals in Norway. Appl. Environ. Microbiol. 46:855-859.

Skirrow, M. B. 1977. Campylobacter enteritis: a "new" disease. Br. Med. J. 2:9-11.

Skirrow, M. B., and J. Benjamin. 1980a. Differentiation of enteropathogenic campylobacter. J. Clin. Pathol. 33:1122.

Skirrow, M. B., and J. Benjamin. 1980b. '1001' Campylobacters: cultural characteristics of intestinal campylobacters from man and animals. J. Hyg. (*Camb.*) 25:427-442

Smibert, R. M. 1974. *Campylobacter*, p.207-212. *In* R. E. Buchanan, and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams and Wilkins Co., Baltimore.

Smibert, R. M. 1978. The genus *Campylobacter*. Annu. Rev. Microbiol. 32:673-709.

Smibert, R. M. 1985. *Campylobacter*., p. 111-118. *In* N. R. Krieg (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams and Wilkins Co., Baltimore.

Smith, T., and M. S. Taylor. 1919. Some morphological and biological characters of the spirilla (*Vibrio fetus*, n. sp.) associated with disease of the fetal membranes in cattle. J. Exp. Med. 30:299-311.

Speelman, P., M. J. Struelens. 1984. *Campylobacter jejuni* in travellers' diarrhea, p. 33-38. *In* J. P. Butzler (ed.), Campylobacter infection in man and animals. CRC Press, Inc., Boca Raton, Fla.

Steele, T. W., and S. N. McDermott. 1984. The use of membrane filters applied directly to the surface of agar plates for the isolation of *Campylobacter jejuni* from feces. Pathol. 16:263-265.

Tanner, E. I., and C. H. Bullin. 1977. Campylobacter enteritis. Br. Med. J. 2:579.

Taylor, D. E., L.-K. Ng, and H. Lior. 1985. Susceptibility of *Campylobacter* species to nalidixic acid, enoxacin, and other DNA gyrase inhibitors. Antimicrob. Agents Chemother. 28:708-710.

Tenover, F. C., J. S. Knapp, C. Patton, and J. J. Plorde. 1985. Use of auxotyping for epidemiological studies of *Campylobacter jejuni* and *Campylobacter coli* infections. Infect. Immun. 48:384-388.

Tenover, F. C., S. Williams, K. P. Gordon, N. Harris, C. Nolan, and J. J. Plorde. 1984. Ultility of plasmid fingerprinting for epidemiological studies of *Campylobacter jejuni* infections. J. Infect. Dis. 149:279.

Thompson, J. S., F. E. Cahoon, and D. S. Hodge. 1986. Rate of *Campylobacter* spp. isolation in three regions of Ontario, Canada, from 1978 to 1985. J. Clin. Microbiol. 24:876-878.

Totten, P. A., C. L. Fennell, F. C. Tenover, J. M. Wezenberg, P. L. Perine, W. E. Stamm and K. K. Holmes. 1985a. *Campylobacter cinaedi* (sp nov.) and *Campylobacter fenelliae* (sp. nov.): Two new *Campylobacter* species associated with enteric disease in homosexual men. J. Infect. Dis. 151:131-139.

Totten, P. A., F. C. Tenover, K. C. S. Chen, K. L. Bruch, K. K. Holmes, and W. E. Stamm. 1985b. Rapid genetic grouping test for identification of *Campylobacter* species. p. 234. *In* A. D. Pearson, M. B. Skirrow, H. Lior and B. Rowe (ed.), Campylobacter III: Proceedings of the third international workshop on campylobacter infections, Ottawa. Public Health Laboratory Service, London.

Trueblood, M. S., and J. O. Tucker. 1957. An improved medium for the cultivation of *Vibrio fetus*. Am. J. Vet. Res. 18:445-448.

Turck, M., 1982. Cephalosporins and related antibiotics: An overview. Rev. Infect. Dis. 4:S281-S287.

Vanhoof, R., M. P. Vanderlinden, R. Dierickx, S. Lauwers, E. Yourassowsky, and J. P. Butzler. 1978. Susceptibility of *Campylobacter fetus* subsp. *jejuni* to twenty-nine antimicrobial agents. Antimicrob. Agents Chemother. 14:553-556.

Vanhoof, R., B. Gordts, R. Dierickx, H. Coignau, and J. P. Butzler. 1980. Bacteriostatic and bactericidal activities of 24 antimicrobial agents against *Campylobacter fetus* subsp. *jejuni*. Antimicrob. Agents Chemother. 18:118-121.

Véron, M., and R. Chatelain. 1973. Taxonomic study of the genus *Campylobacter* Sebald and Véron and designation of the neotype strain for the type species, *Campylobacter fetus* (Smith and Taylor) Sebald and Véron. Int. J. Syst. Bacteriol. 23:122-134.

Walker, R. I., M. B. Caldwell, E. C. Lee, P. Guerry, T. J. Trust, and G. M. Ruiz-Palacios. 1986. Pathophysiology of *Campylobacter* enteritis. Microbiol. Rev. 50:81-94.

Wang, W.-L. L., L. B. Reller, and M. J. Blaser. 1984. Comparison of antimicrobial susceptibility patterns of *Campylobacter jejuni* and *Campylobacter coli*. Antimicrob. Agents Chemother. 26:351-353.

Waterman, S. C., R. W. A. Park, and A. J. Bramley. 1984. A search for the source of *Campylobacter jejuni* in milk. J. Hyg. (*Camb.*) 92:333-337.

Webber, J. A., and T. Yoshida. 1982. Moxalactam: The first of a new class of β-lactam antibiotics. Rev. Infect. Dis. 4:S496-500.

Wesley, R. D., B. Swaminathan, and W. J. Stadelman. 1983. Isolation and enumeration of *Campylobacter jejuni* from poultry products by a selective enrichment method. Appl. Environ. Microbiol. 46:1097-1102.

Whiffen, A. J. 1948. The production, assay, and antibiotic activity of actidione, an antibiotic from *Streptomyces griseus*. J. Bacteriol. 56:283-291.

Wong, K. H., S. K. Skelton, and J. C. Feeley. 1986. Strain characterization and grouping of *Campylobacter jejuni* and *Campylobacter coli* by interaction with lectins. J. Clin. Microbiol. 23:407-410.

Wright, E. P., H. E. Tillett, J. T. Hague, F. G. Clegg, R. Darnell, J. A. Culshaw, and J. A. Sorrell. 1983. Milk-borne campylobacter enteritis in a rural area. J. Hyg. (*Camb.*) 91:227-233.

3. Morphological forms and viability of *Campylobacter* species studied by electron microscopy'

3.1 Introduction

Campylobacter species are gram-negative, non-spore-forming, microaerophilic organisms. *Campylobacter jejuni*, *Campylobacter coli* and *Campylobacter laridis* (Karmali and Skirrow, 1984) have all been implicated in outbreaks of gastroenteritis. Direct examination of fecal specimens by dark field or phase-contrast microscopy is sometimes used as part of the routine diagnostic procedure for *Campylobacter* spp. (Goossens *et al.*, 1984). The spiral shape and darting motility may be used as characteristics to differentiate them from other enteric organisms (Karmali and Skirrow, 1984). Several morphological forms of *Campylobacter* organisms have been reported, including: spirals, S-shapes, gull-shapes, commas, dimpled shapes and coccoid shapes (Karmali and Skirrow, 1984; Merrell *et al.*, 1981; Smibert, 1978). The spiral forms predominate in young cultures, while coccoid forms are found mainly in old cultures (Buck *et al.*, 1983; Ogg, 1962; Pead, 1979; Smibert, 1978; Tritz and Ogg, 1967). The coccoid forms of *Campylobacter fetus* and *C. jejuni* are believed to be degenerative (Buck *et al.*, 1983; Smibert, 1978) and to resemble similar forms in some of the chemoheterotrophic spirilla (Smibert, 1978).

'A version of this chapter has been published by Ng, L.-K., R. Sherburne, D. E. Taylor, and M. E. Stiles. 1985. J. Bacteriol. 164: 338-343.

In this study, single colonies of *C. coli*, *C. jejuni* and *C. laridis* were studied using scanning electron microscopy to demonstrate the transition from spiral to coccoid forms by examining various areas of a colony. Thin sections of *C. jejuni* and *C. laridis* were studied using transmission electron microscopy. The viability of the coccoid forms of *C. jejuni* and *C. coli* was determined by comparative plating of the cultures.

3.2 Materials and methods

3.2.1 Electron microscopy

C. jejuni, *C. coli* and *C. laridis* were grown on Columbia blood agar containing 10% defibrinated sheep blood. Plates were incubated at 37°C in an incubator containing 7% CO_2. The cells were harvested after 48 to 72 h incubation.

For scanning electron microscopy (SEM), agar blocks containing a single colony were cut from the plates and placed in small petri dishes containing 1% (w/v) osmium tetroxide (OsO_4) in veronal buffer (pH 6.1), and held overnight at room temperature to fix the cells in the colony. After fixation, colonies were floated off the agar blocks. The colonies were then dehydrated in a graded alcohol series (25, 50, 75, 90 and 100% ethanol). After critical-point drying the colonies were mounted onto SEM specimen stubs and sputter-coated with gold. Samples were examined with a Cambridge 250 scanning electron microscope.

For transmission electron microscopy (TEM), colonies of *C. jejuni* and *C. laridis* were fixed and dehydrated as described for SEM. After dehydration in ethanol, the specimens were placed in 2 changes of propylene oxide for 15 min each, followed by 30 min in 12% Araldite in propylene oxide, and 1 h in 25% Araldite in propylene oxide. The specimens were then transferred to 50% Araldite in propylene oxide, left in an uncapped vial at room temperature for 24 h, and then transferred to pure Araldite and left for 1 h. The Araldite was replaced and the specimens were cured for 24 h at 60°C. Ultrathin sections (60 nm thick) were prepared using an ultramicrotome (Ultracut, Reichert-Jeny). The sections were placed on 400-mesh copper grids and stained with 5% uranyl acetate in methanol for 10 min and in lead citrate for 4 min.

Suspensions from *C. jejuni* colonies were also studied using TEM by negative-staining with 1% sodium phosphotungstate at pH 7.0. These preparations were made on Formvar and carbon-coated 200-mesh grids. Both the thin sections and the negatively-stained specimens were examined in a Philips EM300 electron microscope.

3.2.2 Viability study

C. coli NCTC 11353 and *C. jejuni* NCTC 11168 were grown in Brucella broth (Difco Laboratories, Detroit, MN, U.S.A.) at 37°C and 7% CO_2 atmosphere for 24 h. Appropriate dilutions of 24-h cultures were plated onto Mueller-Hinton

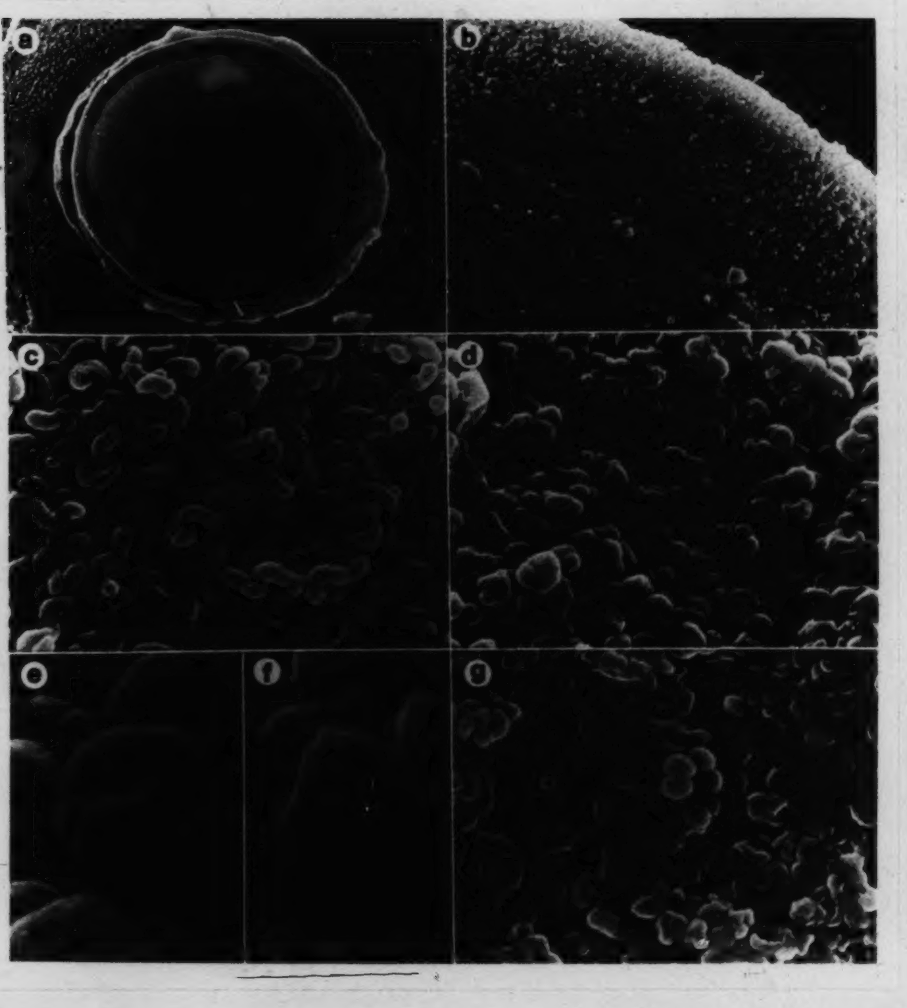
(MH) agar (Oxoid Ltd., Basingstoke, England) so that each plate had about 300 colonies after incubation. After 5, 7 and 10 days of incubation, one of the plates was removed to prepare a cell suspension by transferring sufficient colonies to 0.85% saline to give a turbidity equivalent to the McFarland no. 2 standard. Appropriate dilutions of the saline suspensions were plated in triplicate onto MH to enumerate the CFU/mL of the suspensions. Simultaneously, the saline suspensions were diluted 1:1 or 1:4 with 0.5% phenol. After treating with phenol for 5 min, the cells were non-motile, and the microscopic counts of the different morphological forms were determined using a Petroff-Hausser bacteria counter under phase contrast. The experiment was duplicated for each culture, and the microscopic counts were compared with the CFU/mL on MH agar plates.

3.3 Results

3.3.1 Scanning electron microscopy

Colonies of *Campylobacter* grown for 72 h on Columbia blood agar had a raised center and a narrow, flat edge. The average diameter of the colonies after 72 h of incubation at 37°C was 2 mm. Figure 3.1a shows an entire colony of *C. jejuni* that has been removed from the agar surface and mounted on a SEM specimen stub. Figure 3.1b shows a magnified area of the edge of the colony in Figure 3.1a. In the flat region at the edge of the colony, where actively

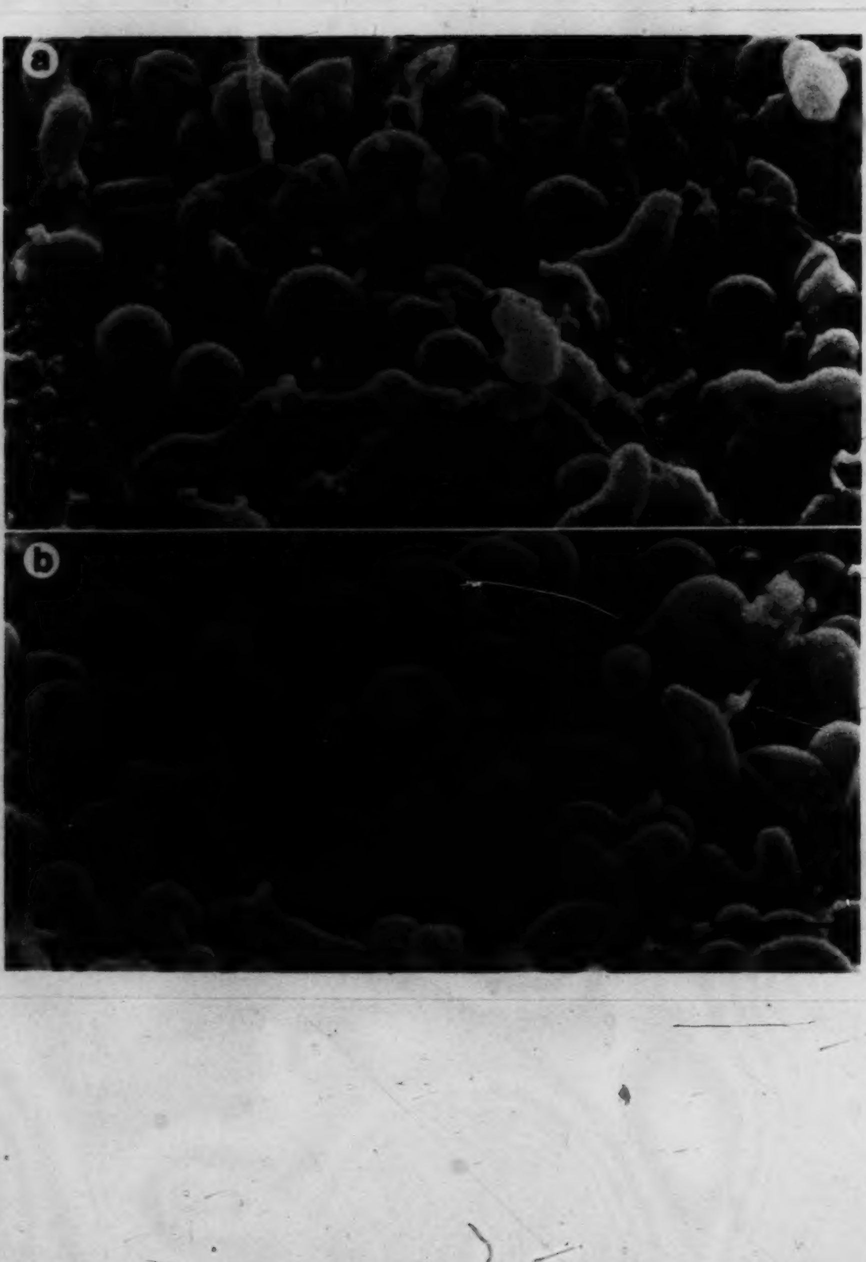
Figure 3.1 Scanning electron micrographs of *C. jejuni* at different magnifications: (a) whole colony, bar represents 200 µm; (b) enlargement of a portion of the edge of the colony (indicated in 1a), bar represents 10 µm; (c) magnification of cells at periphery of colony, bar represents 1 µm; (d) magnification of cells at ridge of colony shown in 1b, bar represents 1 µm; (e) and (f) enlargements of "donut" shaped cells, bar represents 400 nm; (g) magnification of cells at center of colony showing coccus-shaped cells and amorphous material, bar represents 2 µm.


a
b
c
d
e
f
g

growing cells would be expected, the cells were mainly spiral-shaped, as shown in Figure 3.1c. In contrast, cells further from the periphery, at the raised portion (ridge) of the colony, were mainly "ring" or "donut" shaped (Figure 3.1d). The ring- or donut-shaped cells are shown in higher magnification in Figure 3.1e and f, illustrating the hollow center of this form. Cells toward the center of the colony, illustrated in Figure 3.1g, showed a greater predominance of coccoid forms, with an amorphous material on the surface of the colony. Colonies of *C. coli* and *C. laridis* were similar in appearance to *C. jejuni*, except that donut-shaped cells were not observed.

The cells shown in Figures 3.2a and 3.2b were obtained by pouring a layer of purified Agar Noble (Difco) on top of a colony similar to that shown in Figure 3.1a, and then removing the agar layer so that the upper mass of cells was removed. Figure 3.2a, which shows a mixture of spiral and coccoid cells from a *C. coli* colony, represents cells observed at the center of the colony. In fact, the cells are pleomorphic, including S-shaped, gull-shaped and ribbon-shaped spirals, as well as "dimpled" and round coccus forms. In contrast, Figure 3.2b, which shows primarily spiral shaped cells from a colony of *C. jejuni*, represents cells at the periphery of the colony. In this electron micrograph, the end view of a spiral cell may be seen (see arrow in Figure 3.2b). The spirals do not have a ring shape when viewed from one end, due to the small amplitude of the

Figure 3.2 Scanning electron micrographs of *Campylobacter* cells, showing various morphological shapes observed when the surface layers of cells were removed using an agar overlay technique: (a) *C. coli*, toward the center of the colony; and (b) *C. jejuni*, at the periphery of the colony. Bar represents 1 µm.


a
b

helix.

3.3.2 Transmission electron microscopy

Negative-staining of cell suspensions prepared from *C. jejuni* cells grown on Columbia blood agar showed both spiral and coccoid forms (Figure 3.3). Although most coccus-shaped cells appeared to have lost their flagella, some still have flagella attached. Thin sections of colonies of *C. jejuni* (Figure 3.4a) and *C. laridis* (Figure 3.4b to e), viewed by TEM, showed a variety of cell forms. The results for both *C. jejuni* and *C. laridis* were similar. In Figure 3.4a, the colony that had been floated off the agar was sectioned and showed that the cells adjacent to the agar surface were most probably spirals and their cytoplasm was close to the cell wall. The cells further from the agar surface were more likely to be coccus-shaped, and the cytoplasm of these cells was generally separated from the cell wall. Sometimes more than one cytoplasmic mass was observed within a cell envelope (see Figure 3.4b). The cytoplasm of these cells was not as dense as that of the spiral-shaped cells and their cell wall seemed to be stretched, indicating that these cells might be in a degenerative state. In Figures 3.4b and 3.4c, thick bands could be seen, as indicated by the arrows. Serial sections showed that the band was close to the end of the cells. A similar band was observed in all *Campylobacter* strains studied. Some cell types, as seen in Figure 3.4c, have a large diameter and the cytoplasm is separated from

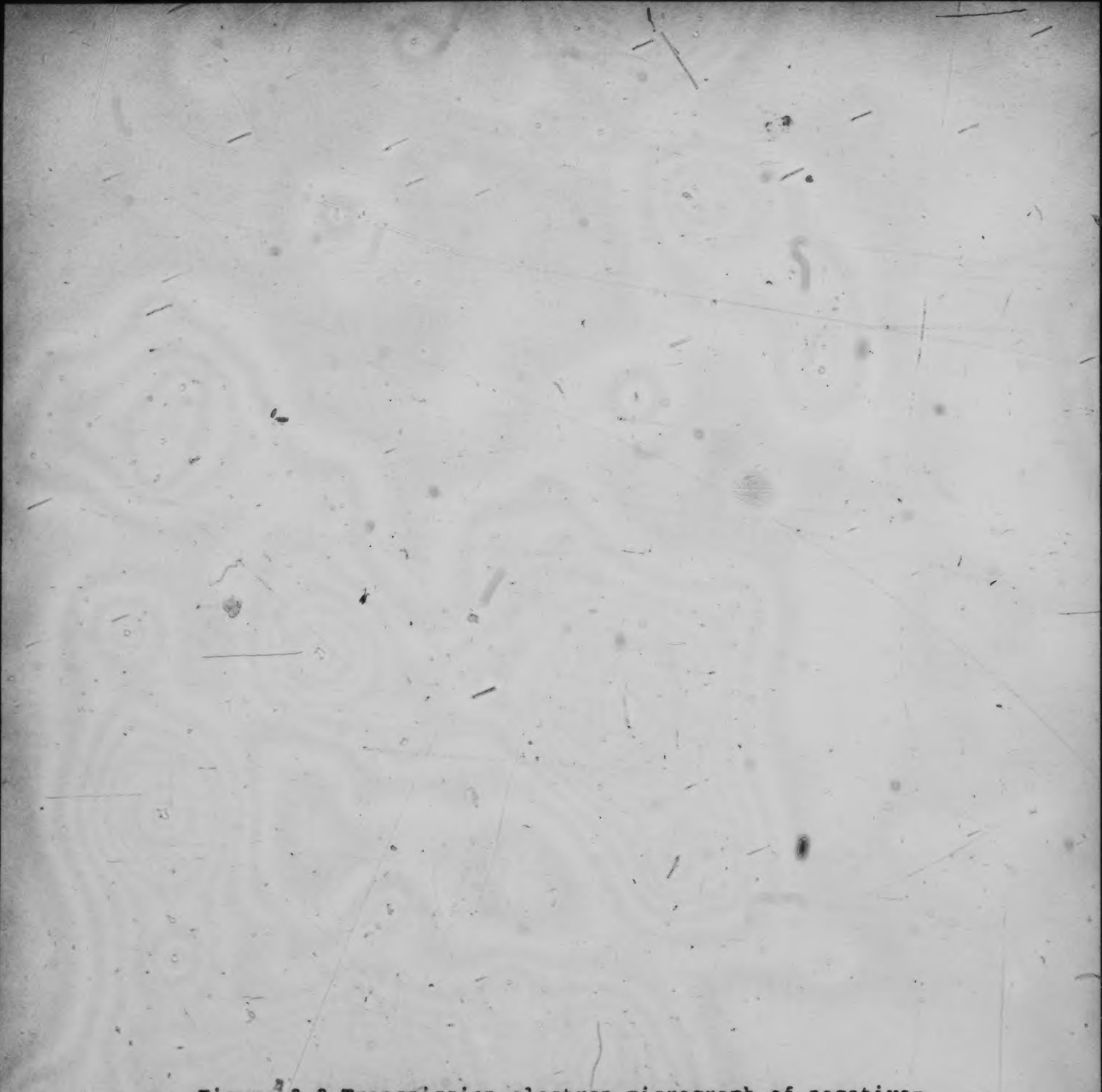


Figure 3.3 Transmission electron micrograph of negative-stained cells of *C. jejuni* showing flagellation of both spiral and coccus-shaped cells. Bar represents 0.5 µm.

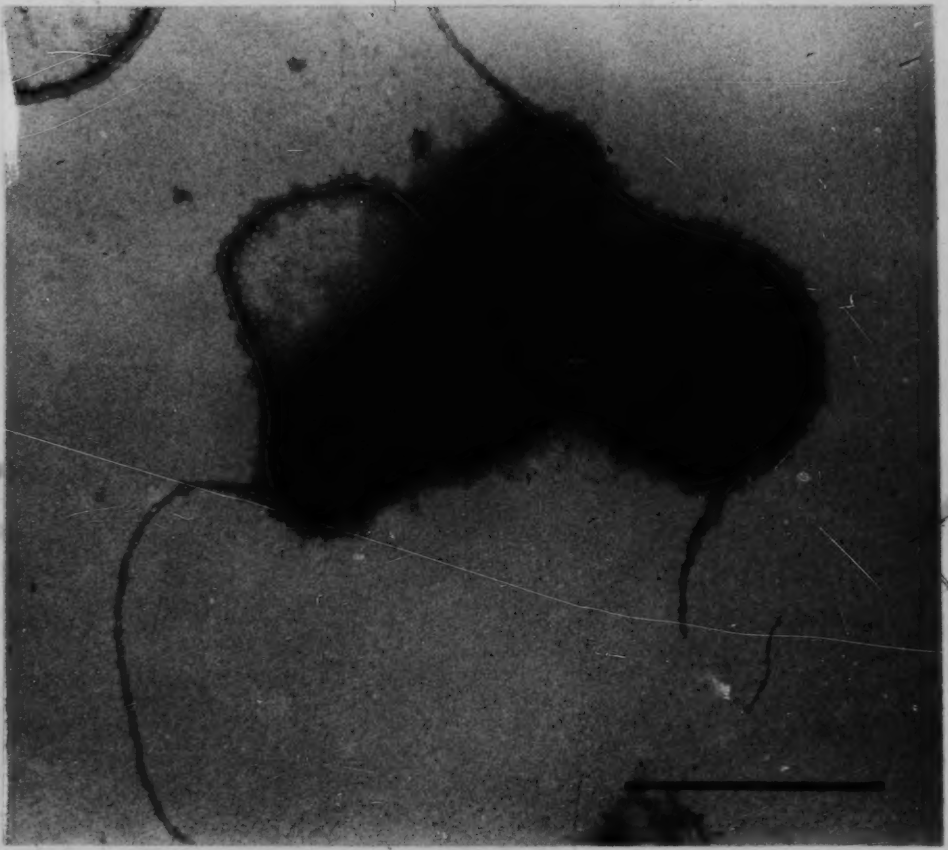
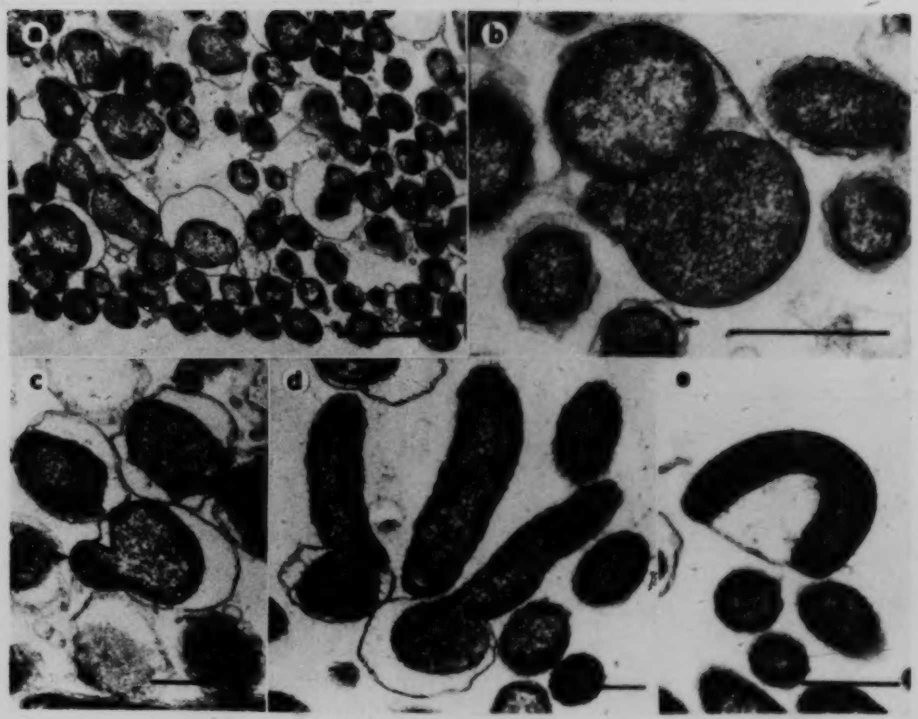


Figure 3.4 Transmission electron micrographs of thin sections of (a) *C. jejuni* and (b) to (e) *C. laridis* showing different morphological forms of the cells. Lower surface of cells in Figure 3.4a was adjacent to agar surface. Bar represents 0.5 µm.


a
b
c
d
e

the cell wall. Most of these cells had a circular shape, indicating that they might be coccoid. Some were club-shaped, as shown in Figure 3.4d, indicating that they might be intermediate forms. Serial sections illustrated that the cytoplasm in the coccoid cells was of variable shape, even though their outline in thin sections was generally circular (see Figure 3.4e).

3.3.3 Viability study

Greater than 99% of ~~the~~ cells of *C. coli* and *C. jejuni* grown in Brucella broth and incubated at 37°C in a modified atmosphere (7% CO_2) for 24 h, were in the spiral form. No coccus-shaped cells were observed under phase contrast. When the 24 h broth culture was diluted and plated onto MH agar, almost 100% of the cells formed colonies (see Table 3.1). Similarly, when *C. coli* and *C. jejuni* were grown on MH agar for 24 h, the predominating cell shape was spiral. After 5 days incubation, the predominant cell shape had shifted to the coccoid form. Plating appropriate dilutions of a suspension prepared from these colonies, which contained 10^6 to 10^7 spiral- and 10^9 coccus-shaped cells per mL, only 10^6 cells formed colonies on MH agar. Both the plate count and the spiral cell count were 1000-fold less than the total microscopic count.

After 7 and 10 days of incubation, colonies were sampled to include cells from the periphery and the center of the colony. Spiral cells were still detected after 10

Table 3.1 Microscopic counts of coccoid and spiral forms of *C. coli* and *C. jejuni* compared with plate counts on Mueller-Hinton agar.

		$\log_{10}$ mean		
		microscopic count		
cultures	no. of replicates	cocci/mL	spirals/mL	plate count (CFU/mL)
C. coli (NCTC 11353)				
24 h Brucella broth culture	1	none detected	8.50	8.41
growth on MH	6*	9.14	6.59	6.46
C. jejuni (NCTC 11168)				
24 h Brucella broth culture	1	none detected	8.60	8.49
growth on MH	6*	9.28	6.90	6.10

* The number of replicates is based on duplicate trials with three samplings (5, 7 and 10 days) from MH agar plates.

days of incubation. The microscopic counts for samples taken after 7 and 10 days ranged from 1.0 to 2.2 x 10^9 coccoid cells and 1.9 to 5.6 x 10^6 spiral-shaped cells. The plate count ranged from 6.7 x 10^5 to 5.1 x 10^6 CFU/mL.

The $\log_{10}$ counts were compared statistically by paired t-test analysis. The $\log_{10}$ mean counts of cell suspensions, containing spiral and coccoid-forms of *C. coli* or *C. jejuni* grown on MH are shown in Table 3.1. The total microscopic counts for both *C. coli* and *C. jejuni* were significantly higher than the plate counts. The number of spiral-shaped cells of *C. coli* enumerated microscopically was the same as the plate count. However, the number of spiral-shaped cells of *C. jejuni* enumerated microscopically was slightly higher than the plate count (P=0.0025), indicating that not all spiral cells formed colonies. However, the difference is less than one log cycle, which may not be of practical importance.

3.4 Discussion

The cells in a single colony are heterogeneous in age and physiological state. It is assumed that at the periphery of the colony the cells are actively growing, while at the center and on the upper surface of the colony nutrients are less available and cells are more likely to be old and inactive. SEM of a single colony of *C. jejuni* showed that cells with characteristic morphological forms predominate at different locations within the colony. Spiral forms predominate at the edge, while coccoid forms predominate in the center, suggesting that these forms represent actively growing and inactive cells, respectively. The donut forms were observed in a small region between the area where the spiral and coccoid forms were observed. This suggests the possibility that they are an intermediate form between spiral and coccus-shaped cells The mechanism of donut formation is not clear, however, other microorganisms such as *Microcyclus* sp. and *Spirosoma* sp. are known to form ring-like structures (Raj, 1977).

Coccoid forms have been reported for other spiral-shaped bacteria, including *Spirillum*, *Vibrio*, *Oceanospirillum* and *Desulfovibrio* spp. (Krieg, 1976). The coccoid forms are believed to be resting stages in *Spirillum* (Krieg and Hylemon, 1976). Reversion of coccoid to spiral forms in *C. fetus* has been reported (Ogg, 1962). However, this reversion was observed when broth or agar cultures, which had less than three spirals per ten fields under microscopic examination (considered to be 100% coccoid forms), were transferred to fresh broth or agar. After 24 to 48 h of incubation, only spiral forms were detected. In contrast, Baker and Park (1975), using a slide cultivation technique, demonstrated that coccoid forms in *Vibrio* spp. were non-viable, and that survival of the culture depended on the few rod forms that were present.

In our study, enumeration of viable cells by plating on MH agar indicated that coccoid cells were probably unable to form colonies. This was supported by our observations from thin sections of *C. jejuni* and *C. laridis* cells. The thin sections of *C. laridis* (Figure 3.4) showed a high incidence of bleb formation. This has been reported by others (Buck *et al.*, 1983; Pead, 1979) to indicate loss of cell wall integrity which represents a degenerative change. The anatomical features that we observed by SEM and TEM were similar to those reported for *C. fetus*, including the presence of thick bands at the flagellated poles (Keeler *et al.*, 1966; Ritchie *et al.* 1966) and the absence of a flagellar sheath (Ferris *et al.*, 1984; McCoy *et al.*, 1975; Ritchie *et al.*, 1966). This suggests that our strains were morphologically similar to other *Campylobacter* strains that have been studied.

It is well documented that all *Campylobacter* exist in different morphological forms. Our studies showed that spiral forms are probably actively growing cells, whereas coccoid forms are old, inactive and possibly degenerative cells. The mechanism of coccoid cell formation remains unknown, but our observation of ring- or donut-shaped cells suggests a progressive change associated with degeneration of the cell wall. The degeneration of the coccoid forms of *C. coli* and *C. jejuni* was indicated in our study by the inability of these cells to form colonies on MH agar. Propagation of cultures, therefore, requires the presence of spiral forms. Without repair of the cell wall, solid media are unlikely to support the growth of coccoid cells. Hence, plate count methods cannot be used for quantitative study of the coccoid cells.

3.5 Bibliography

Baker, D. A., and R. W. A. Park. 1975. Changes in morphology and cell wall structure that occur during growth of *Vibrio* sp. NCTC4716 in batch culture. J. Gen. Microbiol. 86:12-28.

Buck, G. E., K. A. Parshall, and C. P. Davis. 1983. Electron microscopy of the coccoid form of *Campylobacter jejuni*. J. Clin. Microbiol. 18:420-421.

Ferris, F. G., T. J. Beveridge, M. L. Marceau-Day, and A. D. Larson. 1984. Structure and cell envelope associations of flagellar basal complexes of *Vibrio cholerae* and *Campylobacter fetus*. Can. J. Microbiol. 30:322-333.

Goossens, H., M. DeBoeck, H. Van Landuyt, and J. P. Butzler. 1984. Isolation of *Campylobacter jejuni* from human feces, p. 39-50. *In* J. P. Butzler (ed.). Campylobacter infection in man and animals. CRC Press Inc., Boca Raton, Florida.

Karmali, M. A., and M. B. Skirrow. 1984. Taxonomy of the genus *Campylobacter*, p. 1-20. *In* J. P. Butzler (ed.). Campylobacter infection in man and animals. CRC Press Inc., Boca Raton, Florida.

Keeler, R. F., A. E. Ritchie, J. H. Bryner, and J. Elmore. 1966. The preparation and characterization of cell walls and the preparation of flagella of *Vibrio fetus*. J. Gen. Microbiol. 43:439-454.

Krieg, N. R. 1976. Biology of the chemoheterotrophic spirilla. Bacteriol. Rev. 40:55-115.

Krieg, N. R., and P. B. Hylemon. 1976. The taxonomy of the chemoheterotrophic spirilla. Ann. Rev. Microbiol. 30:303-325.

McCoy, E. C., D. Doyle, H. Wiltberger, K. Burda, and A. J. Winter. 1975. Flagellar ultrastructure and flagella-associated antigens of *Campylobacter fetus*. J. Bacteriol. 122:307-315.

Merrell, B. R., R. I. Walker, and J. C. Coolbaugh. 1981. *Campylobacter fetus* ss. *jejuni*, a newly recognized enteric pathogen: morphology and intestinal colonization. Scanning Electron Microscopy 4:125-131.

Ogg, J. E. 1962. Studies on the coccoid form of ovine *Vibrio fetus*. 1. Cultural and serologic investigations. Am. J. Vet. Res. 23:354-358.

Pead, P. J. 1979. Electron microscopy of *Campylobacter jejuni*. J. Med. Microbiol. 12:383-385.

Raj, H. D. 1977. *Microcyclus* and related ring-forming bacteria. CRC Crit. Rev. Microbiol. 5:243-269.

Ritchie, A. E., R. F. Keeler, and J. H. Bryner. 1966. Anatomical features of *Vibrio fetus*: electron microscopic survey. J. Gen. Microbiol. 43:427-438.

Smibert, R. M. 1978. The genus *Campylobacter*. Ann. Rev. Microbiol. 32:673-709.

Tritz, G. J., and J. E. Ogg. 1967. Physical and chemical conditions inducing the coccoid form of ovine *Vibrio fetus*. Am. J. Vet. Res. 28:123-129.

4. Comparison of Basal Media for Culturing *Campylobacter jejuni* and *Campylobacter coli*[1]

4.1 Introduction

Several basal media with various supplements are used for the isolation and growth of *Campylobacter jejuni* and *Campylobacter coli*, such as blood agar base no. 2 (Butzler and Skirrow, 1979; Patton *et al.*, 1981), brucella agar (Acuff *et al.*, 1982; Bowdre *et al.*, 1976; Stern, 1982; Wesley *et al.*, 1983), Columbia blood agar base (Chou *et al.*,1983; Karmali and Fleming, 1979), Mueller-Hinton agar (Lior *et al.*, 1982; Logan and Trust, 1983) and thioglycollate agar medium (Dekeyser, *et al.*, 1972). Common supplements include horse blood (5 to 7%) for use when trimethoprim is incorporated as a selective agent (Bopp *et al.*, 1982; Butzler and Skirrow, 1979) or sheep blood (5-15%) (Butzler and Skirrow, 1979; Doyle and Roman, 1982; Lior *et al.*, 1982; Patton *et al.*, 1981; Rollins *et al.*, 1983), and (or) FBP (a mixture of ferrous sulfate, sodium bisulfite and sodium pyruvate) which is added to increase the aerotolerance of the organisms (Chou *et al.*, 1983; Hoffman *et al.*, 1979a, 1979b). A combination of antibiotics is usually added to inhibit other bacteria present in clinical and environmental samples.

---

[1] A version of this chapter has been published by Ng, L.-K., M. E. Stiles, and D. E. Taylor. 1985. J. Clin. Microbiol. 21:226-230.

For studies of *Campylobacter* cells in pure culture, the use of antibiotic inhibitors is not necessary, and cultures can be incubated at 37 instead of 42°C (Bowdre *et al.*, 1976; Lauwers *et al.*, 1978; Lior *et al.*, 1982). For enumeration of *Campylobacter* cells, it may be necessary to increase levels of agar up to 3% to reduce swarming (Patton *et al.*, 1981, Taylor *et al.*, 1981). Blood is not generally preferred as a supplement to media in research studies because it is undefined, and could cause differences in results between batches. We used eight strains of *C. jejuni* and *C. coli*, of different serotypes, to determine the ability of these organisms to grow on selected basal media, with or without added blood or FBP.

4.2 Materials and methods

4.2.1 Preliminary study

A range of *Campylobacter* organisms including: *C. jejuni*, *C. coli*, *Campylobacter fetus*, and *Campylobacter laridis*, were grown in modified K broth (Taylor *et al.*,1981) containing: tryptic soy broth (Difco Laboratories, Detroit, MN, U.S.A.) 10 g, special peptone (Oxoid) 5 g, yeast extract (Oxoid) 5 g, Tris buffer 0.75 g, sodium pyruvate 5 g in 1 liter of distilled water; and 5 mL of filter sterilized 3% 1,4-dithiothreitol was added per liter. These cultures were incubated at 37°C for 24 h in a modified atmosphere containing 7% CO_2. Appropriate dilutions of these cultures, giving 30 to 300 CFU per plate, were spread onto brucella agar (Difco) and brucella agar prepared according to BBL and Oxoid formulations using appropriate ingredients (BBL and Oxoid); Columbia blood agar base (Oxoid); Columbia blood agar base supplemented with 10% defibrinated sheep blood, with 1.5 or 3% agar; and Mueller-Hinton agar (Difco). Inoculated plates were incubated at 37°C in 7% CO_2 atmosphere and counted after 48 and 72 h of incubation.

4.2.2 Stock cultures

Four strains of *C. jejuni*, serotypes 4, 5, 7 and 17, and four strains of *C. coli*, serotypes 8, 20, 45 and 55, (Lior *et al.*, 1982), were obtained from H. Lior (Laboratory Centre for Disease Control, National Health and Welfare, Ottawa, Canada). The strains were serotyped by H. Lior using the heat-labile serotyping system. All eight strains were human isolates, representing some of the most common serotypes causing human gastroenteritis (Lior, 1984; Lior *et al.*, 1982). Cultures were maintained on Columbia blood agar base (Oxoid) with 10% added defibrinated sheep blood at 37°C in an atmosphere containing 7% CO_2, and they were subcultured twice weekly.

4.2.3 Cultural conditions

All cultures were incubated at 37°C in an atmosphere containing 7% CO_2. For use in the growth studies, a 48-h stock culture on Columbia blood agar with 10% sheep blood was inoculated into 5 mL of modified K broth. Stock cultures were also grown in brucella broth (Difco) for comparison with growth in modified Karmali broth.

4.2.4 Plate counts

Appropriate dilutions of the 24 h cultures grown in modified K broth were prepared in 0.85% saline and surface plated in triplicate onto the following basal media: brucella agar (BBL and Difco), blood agar base no. 2 (Oxoid), campylobacter agar base (Difco), Columbia blood agar base (Difco and Oxoid) and Mueller-Hinton agar (Difco and Oxoid). The composition of the basal media is summarized in Table 4.1. The basal media were also prepared either with addition of FBP (0.05% of each of ferrous sulfate hydrate, sodium metabisulfite and sodium pyruvate), or with 7% defibrinated sheep blood. Plates with FBP were prepoured and held at room temperature (ca. 21°C) to dry overnight. Some media with added sheep blood required additional agar to prevent swarming of the *Campylobacter* organisms. The increased agar levels were as follows: 2% agar in brucella agar (Difco), campylobacter agar base (Difco) and Mueller-Hinton agar (Oxoid), and 2.5% agar in Columbia blood agar base (Oxoid). All prepoured and inoculated plates were held in the dark (Hoffman *et al.*, 1979b). A 0.1 mL sample of appropriate dilutions, to give 30 to 300 CFU per plate, was inoculated onto each medium. Inoculated plates were dried in a laminar flow hood for 15 min, and incubated at 37°C in an

Table 4.1 Composition of basal media used for growth of *Campylobacter* strains.

Ingredients	Brucella agar (BBL and Difco)	Blood agar no. 2 (Difco and Oxoid)[a]	Columbia blood agar (Difco and Oxoid)	Mueller-Hinton agar	
				(Difco)	(Oxoid)
			grams/liter		
"Peptone"[b]	20.0	15.0	23.0		
Yeast extract	2.0	5.0			
Casein hydrolysate[c]				17.5	17.5
Meat infusion				300[d]	6.0
Liver digest		2.5			
Dextrose	1.0				
Starch			1.0	1.5	1.5
NaCl	5.0	5.0	5.0		
Na bisulfite	0.1				
agar	15.0	12.0	15/10[e]	17.0	10.0
pH	7.0	7.4	7.3	7.4	7.4

[a] Difco Campylobacter agar base is a standardized blood agar no. 2.

[b] Brucella agar (BBL) contains 20 g polypeptone peptone, (Difco) contains 20 g of peptamine; Columbia blood agar base (Difco) contains 10 g peptone, 10 g bitone and 3 g tryptic digest of beef heart, (Oxoid) contains 23 g special peptone.

[c] Mueller-Hinton agar (Difco) contains technical casamino acids, (Oxoid) contains casein hydrolysate.

[d] Mueller-Hinton agar (Difco) contains infusion from 300 g of beef.

[e] Columbia blood agar (Difco) contains 15 g Bacto-agar, (Oxoid) contains 10 g agar no. 1.

atmosphere containing 7% CO_2 for 48 and 72 h, for enumeration of the colonies. The experiment was done in duplicate.

4.2.5 Data analysis

Media were compared using $\log_{10}$ transformed data for analysis of variance using a BMDP statistical package (Biomedical Computer Programs, P-series, University of California Press, 1983). Duncan's Multiple Range test was used, where appropriate, to measure differences among media means.

4.3 Results

In the preliminary study, the two *C. laridis* strains grew poorly on Mueller-Hinton agar (Difco) and on brucella agar (Oxoid). All of the strains tested grew poorly on Columbia blood agar base (Oxoid), resulting in a 10- to 1,000-fold reduction in CFU/mL compared with other media. Analysis of variance of the data, excluding the data for Columbia blood agar base and *C. laridis*, revealed a statistically significant difference attributable to media ($P=0.013$). Duncan's Multiple Range test was used at the 95% confidence level to measure differences among media means. The results indicated that brucella agar (Difco) supported a significantly lower CFU/mL than other media. However, the difference in the mean counts was less than five-fold, which is not of practical significance. Furthermore, there was no difference in counts observed on Columbia blood agar base containing 10% defibrinated sheep blood, using the standard 1.0% agar (Oxoid agar no. 1) or with the agar concentration increased to 3%. Colonies on the 1.0% agar medium were flat, large and spreading, whereas on the 3% agar, colonies were very small and difficult to count after 48 h of incubation. Brucella and Mueller-Hinton agars (basal media without added blood) gave counts equivalent to those obtained on Columbia blood agar base with 10% defibrinated sheep blood.

Consequently, the possibility of using basal media without FBP or 7% sheep blood supplement was assessed for quantitative growth of *C. jejuni* and *C. coli*. All strains of *C. jejuni* and *C. coli* grew to 10^8 CFU/mL within 24 h at 37°C in modified K and brucella broths. No change in count was observed after 48 h of incubation. However, after 48 h, phase contrast microscopy revealed that some coccoid forms were present. All strains in this study were grown in modified K broth for 24 h to preclude the degenerative coccoid forms and any possibility that prior growth in brucella broth would give brucella agars an advantage over other basal media. Log_{10} mean counts and the standard deviations for growth of four strains of *C. jejuni* and four strains of *C. coli* on basal and supplemented media are shown in Table 4.2. These data are based on duplicated experiments of the 8 cultures on each medium. An analysis of variance for three grouping factors and repeated measures (BMDP-2V) was used to compare the growth of the 8 test cultures on the

Table 4.2 Effects of growth medium and incubation time on viable counts of *C. jejuni* and *C. coli*.

	Basal medium		with FBP[1]		with Blood[2]	
Medium	48 h	72 h	48 h	72 h	48 h	72 h
	$\log_{10}$ mean CFU/ml ± SD					
Blood agar base no. 2 (Oxoid)	7.02 ±0.97	7.02 ±0.97	8.53 ±0.23	8.56 ±0.20	8.55 ±0.20	8.56 ±0.20
Brucella agar (BBL)	8.51 ±0.23	8.53 ±0.22	8.53 ±0.23	8.53 ±0.23	8.54 ±0.25	8.55 ±0.25
Brucella agar (Difco)	7.88 ±1.24	8.20 ±0.71	8.33 ±0.92	8.58 ±0.21	8.56 ±0.20	8.56 ±0.20
Campylobacter agar base (Difco)	8.24 ±0.91	8.44 ±0.26	8.56 ±0.22	8.54 ±0.22	8.08 ±1.22	8.46 ±0.31
Columbia agar (Difco)	7.20 ±1.11	7.46 ±0.79	8.55 ±0.21	8.57 ±0.21	8.51 ±0.22	8.53 ±0.20
Columbia agar (Oxoid)	6.97 ±1.06	7.15 ±0.97	8.31 ±0.91	8.54 ±0.21	8.06 ±1.22	8.45 ±0.29
Mueller-Hinton (Difco)	8.00 ±1.20	8.40 ±0.28	8.32 ±0.91	8.55 ±0.21	8.27 ±0.90	8.47 ±0.27
Mueller-Hinton (Oxoid)	8.50 ±0.24	8.50 ±0.24	8.34 ±0.91	8.56 ±0.20	8.32 ±0.91	8.52 ±0.22

[1] 0.05% each of ferrous sulfate hydrate, sodium metabisulfite and sodium pyruvate.

[2] 7% defibrinated sheep blood.

8 media, and the effect of added FBP or blood supplements. A summary of the analysis of variance is shown in Table 4.3. Significant effects were attributed to media and supplements. There were also significant interaction effects between culture and supplement, and media and supplement. The interaction effect between media and supplement was probably due to the fact that not all basal media require growth supplements for optimum recovery of the test cultures under the conditions of this experiment. There was a statistically significant increase ($P<0.001$) in the count at 72 h compared with 48 h. Some strains of *C. jejuni* and *C. coli* formed only very small colonies on the basal media after 48 h incubation. After 72 h incubation, all strains of *C. jejuni* and *C. coli* on all media formed colonies with diameters between 1.0 and 2.6 mm. As a result, the 72 h count was selected for further analyses.

The data were separated into three groups, based on the use of growth supplements, for further analysis using a two-way factorial design (ANOVA, BMDP-2V) (Table 4.4). With added supplements (FBP or 7% sheep blood) there was no significant difference between media used for growth of the test organisms, and there was no interaction effect between culture and medium. In contrast, there was a significant difference among basal media without supplements, as well as a significant interaction effect between cultures and media. The $\log_{10}$ mean counts for the cultures on each of the basal media are shown in Table 4.5. The lowest colony counts were

Table 4.3 Summary of repeated measures analysis of variance for growth response of *C. jejuni* and *C. coli* cultures on different growth media after 48 and 72 h incubation.

Source of Variation	Degrees of Freedom	F value	Probability
Culture (C)	7	14.49	<0.001
Media supplements (S)	2	67.68	<0.001
Media (M)	7	10.17	<0.001
CS	14	3.78	<0.001
CM	49	1.28	0.12
SM	14	10.40	<0.001
CSM	98	0.96	0.59
Incubation time (I)	1	20.07	<0.001
CI	7	4.93	<0.001
SI	2	0.21	0.81
MI	7	1.25	0.28
CIS	14	1.60	0.08
CIM	49	0.99	0.50
SIM	14	0.65	0.82
SICM	98	0.59	0.99

Table 4.4 Summary of two-way (factorial design) analyses of variance for comparison of growth response of cultures inoculated on basal and supplemented media.

Source of variation	Degrees of Freedom	F value	Probability
Basal media			
Culture (C)	7	13.20	<0.001
Media (M)	7	35.10	<0.001
CM	49	2.14	0.002
Media with FBP supplement			
Culture (C)	7	19.19	<0.001
Media (M)	7	0.14	0.99
CM	49	0.14	1.00
Media with 7% sheep blood			
Culture (C)	7	18.64	<0.001
Media (M)	7	1.05	0.41
CM	49	0.44	0.99

Table 4.5 Log_{10} mean count of *C. jejuni* and *C. coli* plated on basal media without supplements.

	Media[1]							
Culture	DMH	DCB	DBA	DCAMP	OMH	OCB	OBA2	BBA
C. jejuni								
serotype 4	8.33	7.97	8.64	8.37	8.39	6.81	6.98	8.59
serotype 5	8.56	7.04	8.54	8.56	8.53	7.76	6.98	8.58
serotype 7	8.28	8.16	8.33	8.18	8.28	7.68	7.82	8.27
serotype 17	8.36	6.56	8.45	8.44	8.50	7.18	7.33	8.55
C. coli								
serotype 8	8.18	7.68	8.59	8.28	8.57	7.68	7.60	8.49
serotype 20	8.14	7.07	7.34	8.26	8.23	6.52	5.71	8.36
serotype 45	8.84	8.66	8.81	8.89	8.87	8.18	7.66	8.89
serotype 55	8.48	6.52	6.90	8.55	8.59	5.40	6.10	8.50

[1] Key to Media:

BBA,DBA -- brucella agar (BBL, Difco)

DCAMP ---- campylobacter agar base (Difco)

DCB,OCB -- Columbia blood agar base (Difco, Oxoid)

DMH,OMH -- Mueller-Hinton agar (Difco, Oxoid)

OBA#2 ---- blood agar base no. 2 (Oxoid) observed on Columbia blood agar base (Difco and Oxoid) and blood agar base no. 2 (Oxoid). Two of the *C. coli* strains did not grow well on brucella agar (Difco). However, all strains grew well on other basal media, including campylobacter agar base (Difco), which is equivalent to blood agar base no. 2 (Difco, 1983). With the addition of FBP or 7% defibrinated sheep blood supplements to Columbia blood agar base or blood agar base no. 2 (Oxoid), the inhibitory action of these media was averted. Examination of the means indicates that the interaction effect can be attributed to the variable growth response of the cultures on Columbia blood agar base (Difco and Oxoid), brucella agar (Difco) and blood agar base no. 2 (Oxoid). When these media were excluded from the analysis, there was no significant difference between counts at 48 and 72 h, or between basal media: Mueller-Hinton agar (Difco and Oxoid), campylobacter agar base (Difco) and brucella agar (BBL). The addition of growth supplements to these media did not significantly change the counts of the test cultures on the media.

4.4 Discussion

Modified K broth supported the growth of all *Campylobacter* spp. used in this study. Cultures grew to 10^8-10^9 CFU/mL within 24 h at 37°C. When examined by phase contrast microscopy, all cells appeared to be in the spiral form, and no degenerative cocci were observed. Hence, differences in counts on the solid growth media can be attributed to differences between the media. The nutrient composition of the basal media differs not only between media types but also within types between manufacturers, for example, brucella agar.

The use of 7% CO_2 atmosphere and incubation at 37°C was satisfactory for growth of *Campylobacter* stock strains. Incubation time depends on the medium used, 48 h of incubation is sufficient for media with FBP or blood supplements and for four of the basal media in our study, including Mueller-Hinton agar (Difco and Oxoid), campylobacter agar base (Difco) and brucella agar (BBL). For the other four basal media without supplements, 72 h of incubation is necessary for adequate growth of the colonies.

The low colony counts on the basal media: Columbia blood agar base (Difco and Oxoid), blood agar base no. 2 (Oxoid) and brucella agar (Difco), indicated an inhibition of growth of some cells on these media. This inhibitory effect can be eliminated by addition of either FBP or defibrinated sheep blood supplements to these media. Our studies show that brucella agar (BBL), campylobacter agar base (Difco) and Mueller-Hinton agar (Difco and Oxoid) can be used without enriching supplements for growth of *Campylobacter* cells in pure culture. The recovery rate on these basal media was virtually 100% when compared with supplemented media. In contrast, Bolton and Coates (1983) reported only 9% recovery rate, as the best result with the basal media they used. In their study, the basal media included blood agar base no. 2 (Oxoid) and Columbia blood agar base (Oxoid), which gave the poorest results in our study.

Some researchers increased the amount of agar in *Campylobacter* media, which reduces swarming of the organisms (Patton *et al.*, 1981; Taylor *et al.*, 1981). However, Martin *et al.* (1983) indicated that the use of additional agar (3%) may not be necessary in media used for isolation purposes. The use of additional agar was necessary for some media supplemented with blood to reduce swarming of the organisms on the agar surface. This further detracts from the use of blood as an enriching supplement for routine media intended for enumeration purposes. The strains used in this study did not swarm on basal media (with or without FBP) with normal amounts of agar (1.0% Oxoid and 1.5% Difco). However, the plates were dried, as described in Materials and Methods. Hence, adding extra agar to basal media may not be advantageous because increased agar concentration results in reduced colony size.

For isolation of *Campylobacter* spp. from stools and mixed cultures, antibiotics are added to the media (Butzler and Skirrow, 1979; Lauwers *et al.*, 1978; Martin *et al.*, 1983; Wesley *et al.*, 1983). The medium chosen may affect the final activity of the antibiotics (Butzler and Skirrow, 1979). Mueller-Hinton agar is recommended for antibiotic sensitivity testing (Barry, 1980). We have shown that Mueller-Hinton agar gives high recovery rates when pure cultures are plated onto this medium. It is likely that Mueller-Hinton agar would be the medium of choice for growth of *C. jejuni* and *C. coli*, especially where antibiotics are included in the medium. In subsequent studies, MH agar was used for the growth of *Campylobacter* cultures, and for determining the antibiotic susceptibilities.

4.5 Bibliography

Acuff, G. R., C. Vanderzant, F.A. Gardner, and F. A. Golan. 1982. Evaluation of an enrichment-plating procedure for the recovery of *Campylobacter jejuni* from turkey eggs and meat. J. Food Prot. 45:1276-1278.

Barry, A. L. 1980. Procedure for testing antibiotics in agar media: theoretical considerations, p.1-23. *In* V. Lorian (ed.), Antibiotics in laboratory medicine. Williams and Wilkins Co., Baltimore.

Bolton, F. J., and D. Coates. 1983. Development of a blood-free *Campylobacter* medium: screening tests on basal media and supplements, and the ability of selected supplements to facilitate aerotolerance. J. Appl. Bacteriol. 54:115-125.

Bopp, C. A., J. G. Wells, and T. J. Barrett. 1982. Trimethoprim activity in media selective for *Campylobacter jejuni*. J. Clin. Microbiol. 16:808-812.

Bowdre, J. H., N. R. Krieg, P. S. Hoffman, and R. M. Smibert. 1976. Stimulatory effect of dihydroxyphenyl compounds on the aerotolerance of *Spirillum volutans* and *Campylobacter fetus* subspecies *jejuni*. Appl. Environ. Microbiol. 31:127-133.

Butzler, J. P., and M. B. Skirrow. 1979. Campylobacter enteritis. Clin. Gastroenterol. 8:737-765.

Chou, S. P., R. Dular, and S. Kasatiya. 1983. Effect of ferrous sulfate, sodium metabisulfite, and sodium pyruvate on survival of *Campylobacter jejuni*. J. Clin. Microbiol. 18:986-987.

Dekeyser, P., M. Gossuin-Detrain, J. P. Butzler, and J. Sternon. 1972. Acute enteritis due to related vibrio: First positive stool cultures. J. Infect. Dis. 125:390-392.

Difco Laboratories. 1983. Technical Information: media for the primary isolation of *Campylobacter*. Difco Laboratories, Detroit.

Doyle, M. P., and D. J. Roman. 1982. Response of *Campylobacter jejuni* to sodium chloride. Appl. Environ. Microbiol. 43:561-565.

Hoffman, P. S., H. A. George, N. R. Krieg, and R. M. Smibert. 1979a. Studies of the microaerophilic nature of *Campylobacter fetus* subsp. *jejuni*. II. Role of exogenous superoxide anions and hydrogen peroxide.

Can. J. Microbiol. 25:8-16.

Hoffman, P. S., N. R. Krieg, and R. M. Smibert. 1979b. Studies of the microaerophilic nature of *Campylobacter fetus* subsp. *jejuni*. I. Physiological aspects of enhanced aerotolerance. Can. J. Microbiol. 25:1-7.

Karmali, M. A., and P. C. Fleming. 1979. Campylobacter enteritis in children. J. Pediatr. 94:527-533.

Lauwers, S., M. De Boeck, and J. P. Butzler. 1978. Campylobacter enteritis in Brussels. Lancet i:604-605.

Lior, H. 1984. Serotyping of *Campylobacter jejuni* and *Campylobacter coli* by slide agglutination based on heat-labile antigenic factors, p. 61-76. *In* J.-P. Butzler (ed.), Campylobacter infection in man and animals. CRC Press, Inc., Boca Raton, Fla.

Lior, H., D. L. Woodward, J. A. Edgar, L. J. Laroche, and P. Gill. 1982. Serotyping of *Campylobacter jejuni* by slide agglutination based on heat-labile antigenic factors. J. Clin. Microbiol. 15:761-768.

Logan, S. M., and T. J. Trust. 1983. Molecular identification of surface protein antigens of *Campylobacter jejuni*. Infect. Immun. 42:675-682.

Martin, W. T., C. M. Patton, G. K. Morris, M. E. Potter, and N. D. Puhr. 1983. Selective enrichment broth medium for isolation of *Campylobacter jejuni*. J. Clin. Microbiol. 17:853-855.

Patton, C. M., S. W. Mitchell, M. E. Potter, and A. F. Kaufmann. 1981. Comparison of selective media for primary isolation of *Campylobacter fetus* subsp. *jejuni*. J. Clin. Microbiol. 13:326-330.

Rollins, D. M., J. C. Coolbaugh, R. I. Walker, and E. Weiss. 1983. Biphasic culture system for rapid *Campylobacter* cultivation. Appl. Environ. Microbiol. 45:284-289.

Stern, N. J. 1982. Selectivity and sensitivity of three media for recovery of inoculated *Campylobacter fetus* ssp. *jejuni* from ground beef. J. Food Safety 4:169-175.

Taylor, D. E., S. A. De Grandis, M. A. Karmali, and P. C. Fleming. 1981. Transmissible plasmids from *Campylobacter jejuni*. Antimicrob. Agents Chemother. 19:831-835.

Wesley, R. D., B. Swaminathan, and W. J. Stadelman. 1983. Isolation and enumeration of *Campylobacter jejuni* from poultry products by a selective enrichment method. Appl. Environ. Microbiol. 46:1097-1102.

5. Inhibition of *Campylobacter coli* and *Campylobacter jejuni* by antibiotics used in selective growth media[1]

5.1 Introduction

Campylobacter jejuni and *Campylobacter coli* are now recognized as important agents of gastroenteritis. In 1972, a filtration technique for isolation of *Campylobacter* from stool samples was described (Butzler *et al.*, 1973; Dekeyser *et al.*, 1972). In 1977, Skirrow simplified the isolation procedure by using solid media containing three antibiotics (trimethoprim, polymyxin B sulfate and vancomycin) for primary isolation of *Campylobacter* organisms. Subsequently, other antibiotic combinations have been proposed as selective agents to recover *C. jejuni* and *C. coli* from various sources (Blaser *et al.*, 1979, Bolton and Robertson, 1982; Dekeyser *et al.*, 1972; Goossens *et al.*, 1983; Karmali and Fleming, 1979; Lauwers *et al.*, 1978; Rosef *et al.*, 1983; Wesley *et al.*, 1983). Besides differences in the types and concentrations of antibiotics used, the basal media used with the antibiotics also differ (Butzler and Skirrow, 1979) giving rise to the possibility of inconsistent results between laboratories.

There are many reports of the antibiograms of *C. jejuni*, including a review by Vanhoof (1984). The minimal inhibitory concentrations (MIC) of antibiotics used in many of the selective media have also been reported for *C. jejuni* (Ahonkai *et al.*, 1981, Dekeyser *et al.*, 1972; Karmali *et al.*, 1980; Karmali *et al.*, 1981; Michel *et al.*, 1983; Ringertz *et al.*, 1981; Walder, 1979; Vanhoof, 1978). However, there are far fewer reports of antibiograms for *C. coli*. This is probably due to the lower isolation frequency of this organism from patients (Karmali and Skirrow, 1984). In the past, *C. jejuni* and *C. coli* were not differentiated, but were classified as *C. fetus* subsp. *jejuni* (Karmali and Skirrow, 1984). Information on antibiotic susceptibility for development of media was based primarily on antibiotic resistance of *C. jejuni*. Selective media for the isolation of *Campylobacter* may be more inhibitory to *C. coli* than *C. jejuni*. This inhibitory effect against *C. coli* might be even more significant where media are used for enumeration purposes, for example in foods (Stern, 1982).

---

[1] A version of this chapter has been published by Ng, L.-K., M. E. Stiles, and D. E. Taylor. 1985. J. Clin. Microbiol. 22:510-514.

In this study, the inhibition of *C. coli* by antibiotics used in media selective for *Campylobacter* spp. was compared with the inhibition of select strains of *C. jejuni*. This was done by determining the MICs for a range of antibiotics used in selective media, as well as by comparing the effect of the various antibiotic combinations used in *Campylobacter* media. Antibiotic combinations were evaluated by comparing plate counts on a standard basal medium with and without antibiotics.

5.2 Materials and methods

5.2.1 Cultures

A total of 24 *C. coli* strains was selected from our collection for comparison of their antibiotic resistance with that of 6 *C. jejuni* strains. All of these strains were from human and animal sources. Reference strains were included from type culture collections: *C. coli* CIP 7077 and CIP 7080, *C. jejuni* NCTC 11353 and NCTC 11392. In addition, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* (Oxford strain), *Streptococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, *Campylobacter fetus* subsp. *fetus* ATCC 27374, and *Campylobacter laridis* NCTC 11352 were used as reference cultures for MIC determinations. All *Campylobacter* strains were stored at -70°C in a preservation medium containing glycerol (40%) and trisodium citrate (3%). They were subcultured on Mueller-Hinton (MH) agar before MIC determination. Other species were subcultured in a similar manner on nutrient agar.

5.2.2 Minimal Inhibitory Concentration (MIC) determination

A colony of *Campylobacter* was selected from a 48-h MH plate and inoculated into 2 mL of MH broth. The inoculated broth was incubated overnight at 37°C in an atmosphere containing 7% CO_2. Cultures were diluted with Penassay broth (Difco Laboratories, Detroit, MN, U.S.A.) to give approximately 10^7 CFU/mL, so that 10^4-10^5 CFU would be inoculated onto antibiotic plates (Barry, 1980) with a Steers replicator (Steers *et al.*, 1959). Reference cultures, other than the *Campylobacter* reference strains, were grown in nutrient broth at 37°C, diluted and treated in the same manner as the *Campylobacter* strains.

Antibiotics included in the MIC determinations were bacitracin, cephalothin, colistin, polymyxin B sulfate, novobiocin, rifampicin, trimethoprim and vancomycin (all supplied by Sigma Chemicals, St. Louis, MO, U.S.A.). MICs were determined on MH agar containing two-fold increases in concentrations of antibiotics. All antibiotic plates were used within 2 days of preparation. Plates containing rifampicin and novobiocin were stored in the dark. The MIC was defined as the lowest concentration of the antibiotic at which there was complete inhibition of growth.

5.2.3 Effect of antibiotic combinations used in selective media

Campylobacter strains were selected from the cultures used in the MIC determinations to provide a range of *C. coli* strains with different antibiotic resistance patterns. In addition, three *C. jejuni* strains were also included as a reference for the *C. coli* results. The strains were incubated in brucella broth, incubated at 42°C in anaerobic jars containing a modified atmosphere of 5% O_2, 10% CO_2 and 85% N_2. The 24-h brucella broth cultures were examined by phase contrast microscopy to ensure that the the cells were predominantly in the spiral-form, as opposed to the coccoid-form. MH agar was used as the basal medium for this study, because of our previous observation that this medium supported maximum recovery of *Campylobacter* cells in the absence of blood or growth supplements (Ng *et al.*, 1985, see Chapter 4). The antibiotic combinations listed in Table 5.1 were added to MH agar. The 24-h cultures were diluted with 0.85% saline bacause *Campylobacter* cells suspended in this diluent for up to 60 min retained their viability when plated onto MH agar. Appropriate dilutions were inoculated in triplicate onto each agar medium and spread over the agar surface with a sterile glass "hockey stick". Plates with 30 to 300 colonies were counted. The differences in counts represented differences in inhibitory effect of the antibiotic combination. Inoculated plates were incubated at 42°C for 48 h in modified atmosphere containing 5% O_2, 10% CO_2 and 85% N_2 in plastic bags containing glycerol (Stern, 1982). Plates with no colonies or colonies that were too small to count after 48 h incubation were reincubated for an additional 48 h.

Initially, neither blood nor growth supplements were added, except to media M5 and M6. These media were prepared with and without blood because they contained novobiocin which is known to be bound by serum protein (Barry, 1980). Therefore, the addition of blood may reduce the inhibitory effect of novobiocin in these media. Furthermore, the agar concentration of these media was increased to 2% to prevent

Table 5.1 Antibiotic composition of selective media used for quantitative comparisons of effects of antibiotics on the growth of *C. coli* and *C. jejuni*.

	Antibiotics[a] concentrations per liter of medium									
Media[b]	TMP mg	Van mg	PB IU	CL IU	RA mg	AC mg	CF mg	AmB mg	B IU	NB mg
M1	5	10	2,500							
M2	5	10	5,000							
M3	5	10	2,500				15	2		
M4	10		5,000		10	100				
M5			10,000			50			25,000	5
M6				10,000		50	15		25,000	5
M7			20,000		25		6.25			
M8				10,000	10		15	2		
M9				10,000			15	1		

[a] TMP trimethoprim; Van vancomycin; PB polymyxin B sulfate; CL colistin; RA rifampicin; AC actidione; CF cephalothin; AmB amphotericin B; B bacitracin; NB novobiocin.

[b] M1 Skirrow, 1977; M2 Karmali and Fleming, 1979; M3 Blaser *et al.*, 1979; M4 Bolton and Robertson, 1982; M5 Dekeyser *et al.*, 1972; M6 Lauwers *et al.*, 1978; M7 Wesley *et al.*, 1983; M8 Goossens *et al.*, 1983; M9 Rosef *et al.*, 1983.

swarming of the cells. Subsequently, media that were inhibitory to *C. coli* or *C. jejuni* were retested with growth supplements (Wesley *et al.*, 1983) or sheep or lysed horse blood added at recommended levels (Blaser *et al.*, 1979, Bolton and Robertson, 1982, Dekeyser *et al.*, 1972; Goossens *et al.*, 1983, Karmali and Fleming, 1979, Rosef *et al.*, 1983, Skirrow, 1977).

5.3 Results

5.3.1 MIC determinations

All cultures grew well on MH agar without antibiotics. MICs for *E. coli*, *S. aureus*, *S. faecalis* and *P. aeruginosa* were within the ranges for cephalothin, colistin, polymyxin, trimethoprim and vancomycin (Gavan and Barry, 1980; Washington and Sutter, 1980), and gave consistent results between trials. MICs for the reference *Campylobacter* strains used in this study were within the range of expected values (Vanhoof, 1984). The MIC data shown in Table 5.2 represent the ranges for 30 strains of *Campylobacter*. The results are based on data obtained after 24 h of incubation. After 48 h of incubation, MICs were 1 to 2 dilutions higher for some antibiotics, notably cephalothin, colistin, polymyxin and rifampicin. All test strains of *C. jejuni* were resistant to the antibiotics at levels used in selective media. The MICs of bacitracin, trimethoprim and vancomycin for test and reference strains of *C. coli* were similar to those for *C.*

Table 5.2 MIC of eight antibiotics against 24 strains of *C. coli* and 6 strains of *C. jejuni*.

Antibiotics	*C. coli* Range	*C. coli* MIC_{50}[a]	*C. coli* MIC_{90}[b]	*C. jejuni* Range
	µg/mL			
Bacitracin[e]	>512	>512	>512	>512
Cephalothin	8->256	256	>256	128->256
Colistin[d]	2-32	8	16	4-32
Novobiocin	1-128	64	128	32-64
Polymyxin B[c]	0.5-8	2	8	2-8
Rifampicin	2->128	32	64	32-128
Trimethoprim	128-256	256	256	256
Vancomycin	>128	>128	>128	>128

[a] MIC_{50} - The concentration of antibiotic that inhibited 50% of the cultures.

[b] MIC_{90} - The concentration of antibiotic that inhibited 90% of the cultures.

[c] polymyxin B -- 1 µg/mL = 8 IU

[d] colistin ----- 1 µg/mL = 13.6 IU

[e] bacitracin --- 1 mg/mL = 65.2 IU

jejuni. The resistance of *C. coli* to other antibiotics varied over a greater range than that of *C. jejuni* strains tested.

Based on the MIC data, a total of seven *C. coli* strains was selected for further study. The MICs are shown in Table 5.3, except for bacitracin, trimethoprim, and vancomycin, which had MICs equivalent to those for *C. jejuni*. Three *C. jejuni* strains were also selected for comparison with the the *C. coli* strains. Their antibiotic resistance was as follows: trimethoprim 256 µg/mL, vancomycin >128 µg/mL, polymyxin B sulfate 4 to 8 µg/mL, colistin 8 to 16 µg/mL, rifampicin 32 to 128 µg/mL, cephalothin 128 µg/mL, bacitracin >512 µg/mL, and novobiocin 16 to 32 µg/mL.

5.3.2 Effect of antibiotic combinations

The resistance of the selected *Campylobacter* strains to the combinations of antibiotics used in selective media was compared in MH agar. The number of colonies (CFU/mL) growing on MH agar was compared with the number of colonies growing on MH with added antibiotic combinations. The differences in $\log_{10}$ CFU/mL on MH and MH with antibiotics for each test culture with each antibiotic combination are shown in Table 5.4. The three *C. jejuni* strains grew well in the presence of the antibiotic combinations, except on media M5, M6 and M7. The greatest level of inhibition was a 5 log cycle decrease in count for *C. jejuni* UA526 on medium M5. The addition of blood to media M5 and M6 reduced, but did not

Table 5.3 Susceptibility (MIC) of selected strains of *C. coli* to polymyxin, colistin, rifampicin, cephalothin and novobiocin.

Cultures	Antibiotic[a]				
	PB	CL	RA	CF	NB
	MIC (μg/mL)				
UA37	8	8-16	64-128	>256	128
UA40	2	8	2-4	64	2
UA44	0.5-2	4	16-32	128	32-64
UA100	8	8	16-32	128	64
UA420	2-4	16-32	32-64	>256	64
UA421	2-4	16	≤1-2	4-8	≤1
UA530	8	8-16	32-64	128-256	64

[a] PB: polymyxin B sulfate;
CL: colistin;
RA: rifampicin;
CF: cephalothin;
NB: novobiocin.

Table 5.4 Inhibitory effect of antibiotic combinations added to Mueller-Hinton agar against *C. coli* and *C. jejuni*.

	Differences in $\log_{10}$ CFU/mL on selective media compared with MH[1]										
Cultures	M1	M2	M3	M4	M5	M5B	M6	M6B	M7	M8	M9
C. coli											
UA37	<1	<1	<1	<1	5	<1	<1	<1	>7	<1	<1
UA40	<1	<1	<1	7	>6	>6	>6	>6	>6	>6	>1
UA44	<1	4	<1	>7	>7	<1	1	<1	>7	3	<1
UA100	<1	<1	<1	5	5	<1	<1	<1	>7	2	<1
UA420	<1	<1	<1	2	1	<1	<1	<1	7	<1	<1
UA421	<1	<1	>7	6	>7	>7	>7	>7	>7	>7	>7
UA530	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
C. jejuni											
UA1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
UA124	<1	<1	<1	<1	<1	<1	2	<1	1	<1	<1
UA526	<1	<1	<1	<1	5	3	2	1	3	<1	<1

---

[1] $\log_{10}$ count on MH minus $\log_{10}$ count on selective medium, corrected to the nearest log difference.

Abbreviations for selective media: M1 Skirrow, 1977; M2 Karmali and Fleming, 1979; M3 Blaser *et al.*, 1979; M4 Bolton and Robertson, 1982; M5 Dekeyser *et al.*, 1972; M5B M5 with sheep blood; M6 Lauwers *et al.*, 1978; M6B M6 with sheep blood; M7 Wesley *et al.*, 1983; M8 Goossens *et al.*, 1983; M9 Rosef *et al.*, 1983.

eliminate the inhibitory effect.

The *C. coli* strains grew well on medium M1. In comparison with *C. jejuni*, however, they were inhibited by some antibiotic combinations. Medium M7 caused the greatest inhibition. Counts for 6 of the 7 strains were reduced by more than 7 log cycles. The cephalothin-susceptible strain (UA421) was inhibited by most antibiotic combinations. The addition of 10% sheep blood to media M5 and M6 reduced the inhibitory effect for some strains, but failed to do so for others. For example, the virtual elimination of the inhibitory effect of medium M5 on strains UA37 and UA44 compared with no reduction in effect on UA40 and UA421.

As medium M7 was so inhibitory to the test strains, the effect of 10% sheep blood added to the medium was studied. For those strains that exhibited intermediate to low levels of inhibition (1 to 4 log cycles), the addition of 10% sheep blood markedly reduced the inhibitory effect. For those strains that showed high levels of inhibition (7 log cycles), the addition of blood reduced the level of inhibition by 1 to 2 log cycles for strains UA37 and UA420, but there was less than one log cycle reduction of inhibition for other strains. Medium M7 was originally formulated with hemin (2 mg/L) and 0.025% of each of ferrous sulfate, sodium metabisulfite, and sodium pyruvate (FBP) (Wesley *et al.*, 1983). Medium M7 was retested with these growth supplements instead of 10% sheep blood. Even though the counts were improved with the addition of hemin and FBP, five of the seven *C. coli* strains still had counts that were 3 to 5 log cycles less than MH. The antibiotic combination used in medium M7 was more inhibitory to *C. coli* than *C. jejuni*.

Strains that were inhibited by the antibiotic combinations (see Table 5.4) were plated onto supplemented media for further evaluation using sheep blood or lysed horse blood in accordance with recommendations for the selective media (Blaser *et al.*, 1979; Bolton and Robertson, 1982; Goossens *et al.*, 1983; Karmali and Fleming, 1979; Rosef *et al.*, 1983; Skirrow, 1977). The addition of blood to media M3, M4, M8 and M9 did not change the inhibitory effect of the antibiotic combinations in these media against UA421. However, for other strains of *C. coli*, the addition of blood to media that caused reduced recovery of *C. coli* strains resulted in improved recovery of 2 to 5 log cycles. The addition of blood to medium M4 yielded variable results with strains UA40, UA44, and UA100. Up to seven replicates were done with these strains on M4 to study this variability. The mean $\log_{10}$ CFU/mL and the standard deviations for strains UA40, UA44 and UA100 are shown in Table 5.5. The standard deviations of counts on medium M4 with blood were higher than either MH agar or medium M4. Two batches of blood were not as effective as the others in improving the count on medium M4. Strain UA40 showed great variation in growth response on medium M4 with and without blood.

Table 5.5 Mean $\log_{10}$ CFU of *C. coli* strains UA40, UA44 and UA100 plated onto MH, M4 and M4 with blood.

Strains of *C. coli*	Mean $\log_{10}$ CFU/mL ± SD[a] MH[b]	 M4[c]	 M4+Blood
UA40	8.26 ± 0.28	3.66 ± 2.98	5.14 ± 2.85
UA44	8.21 ± 0.12	2.05 ± 0.42	6.44 ± 1.86
UA100	9.14 ± 0.07	3.52 ± 1.01	7.33 ± 1.58

[a] SD: standard deviation

[b] MH: Mueller-Hinton agar

[c] M4: Bolton and Robertson, 1982

5.4 Discussion

Primary isolation media should not be excessively inhibitory. However, for isolation as opposed to enumeration of bacteria, greater degrees of inhibition can be tolerated. Media used for the isolation of *Campylobacter* spp. depend on a range of antibiotics for their selective effect. This is necessary to control the competing microorganisms in stool specimens. In water and foods, in which *Campylobacter* spp. are likely to be present in low numbers compared with other organisms, a similar if not more critical selection process is necessary.

The MICs of eight antibiotics used in the selective media (see Table 5.1) were determined. Amphotericin B and actidione (cycloheximide) were excluded because they are added for their fungicidal effect, and are not expected to have an effect on bacteria (Berger, 1982; Whiffin, 1948). Cephalothin was substituted for other cephalosporins used in the original formulations (Goossens *et al*., 1983; Wesley *et al*., 1983). The susceptibility of *Campylobacter* to the cephalosporins used in selective media is similar (Ahonkai *et al*., 1981; Karmali *et al*., 1981; Walder, 1979), hence this substitution should not affect the results. Saku *et al*. (1983) observed no difference between MH with and without blood when the susceptibility of *Campylobacter* strains to 12 antibiotics was tested. However, serum proteins bind to some antibiotics, such as novobiocin (Barry, 1980; Gebhart, *et al*., 1985, Karmali *et al*., 1981). This may account for the lower MICs for novobiocin observed on MH agar in this study. The MICs for trimethoprim determined on blood-free MH in this study were similar to other reports (Karmali *et al.*, 1981; Michel *et al.*, 1983; Ringertz *et al.*, 1981).

MICs for *C. jejuni* are generally determined after 48 h of incubation (Ahonkai *et al.*, 1981; Walder, 1979; Vanhoof *et al.*, 1978). In fact, MICs are generally recommended to be read as soon as adequate growth is observed on control plates (Waterworth, 1978; Williams and Leung, 1978). Incubation for 48 h gave higher MIC's for cephalothin, colistin and polymyxin. Prolonged incubation is not recommended for colistin, polymyxin and rifampicin (Williams and Leung, 1978). Using 24 h of incubation, results for the reference strains generally fell within the range of MICs reported in the review by Vanhoof (1984). Even though most strains of *C. coli* were resistant to the individual antibiotics used in the selective media, their combined effect often reduced the recovery of viable cells. The MICs of novobiocin and rifampicin for the cephalothin-susceptible strain of *C. coli* used in this study (UA421) were also lower than the parent strain (UA420). About 2% of *C. coli* are reported to be cephalothin-susceptible (Karmali and Skirrow, 1984).

The combination of antibiotics used in Skirrow's medium (M1), polymyxin B sulfate, trimethoprim and vancomycin, had the least inhibitory effect on the *C. coli* and *C. jejuni* strains. In contrast, the most inhibitory medium (M7) contained cephalothin, polymyxin B sulfate and rifampicin. The concentration of polymyxin in medium M7 is eight times that used in M1. Lovett *et al.* (1983) reported that high levels of polymyxin were inhibitory to many *C. jejuni* strains in their collection. Media that use colistin in place of polymyxin B, especially medium M6 in which a high concentration of bacitracin (25,000 IU/L) was also used, inhibited growth of *C. coli* strains. This might be expected since polymyxin B and colistin are related antibiotics with similar modes of action (Kucer and Bennett, 1975). Other antibiotic combinations had an intermediate effect against *C. coli*, between that observed for media M1 and M7.

Added blood or growth supplements did not eliminate the inhibitory effect on the *C. coli* strains. This supported the hypothesis that growth of *C. coli* may be inhibited on media selective for *Campylobacter* spp. This could account for the lower incidence of isolation of *C. coli* from stool specimens obtained from patients with gastroenteritis. Although the addition of blood to some media reduced or eliminated the inhibitory effect of the antibiotic combinations, blood can cause variable and misleading results. This was seen with medium M4 in which unaccountably poor recovery of cells was observed with two batches of blood used. This effect is convincing evidence for the desirability of developing or adapting a selective medium for *Campylobacter* spp. that does not rely on blood as an ingredient.

This study has indicated the possibility that some media used for detection of *Campylobacter* spp. might inhibit the growth of *C. coli*. The antibiotic combinations used to suppress growth of competing microorganisms in clinical specimens inhibit up to 10^7 *C. coli* cells that have been grown in synthetic media. Although the strains used in this study might not represent isolates from clinical specimens, it is likely that they indicate a problem for enumerating or isolating *C. coli* from the extra-enteral environment, such as water and foods.

5.5 Bibliography

Ahonkhai, V. I., C. E. Cherubin, M. F. Sierra, V. D. Bokkenheuser, M. A. Shulman, and A. C. Morsenthal. 1981. In vitro susceptibility of *Campylobacter fetus* subsp. *jejuni* to *N*-formimidoyl thienamycin, rosaramicin, cefoperazone, and other antimicrobial agents. Antimicrob. Agents Chemother. 20:850-851.

Barry, A. L. 1980. Procedure for testing antibiotics in agar media: Theoretical considerations, p. 1-23. *In* V. Lorian (ed.), Antibiotics in laboratory medicine. The William & Wilkins Co., Baltimore.

Berger, J. 1982. Antibiotics (polyenes), p. 275-300. *In* M. Grayson (ed.), Antibiotics, chemotherapeutics, and antibacterial agents for disease control. John Wiley and Sons, N. Y.

Blaser, M. J., I. D. Berkowitz, F. M. LaForce, J. Cravens, L. B. Reller, and W.-L. L. Wang. 1979. Campylobacter enteritis: Clinical and epidemiologic features. Ann. Intern. Med. 91:179-185.

Bolton, F. J., and L. Robertson. 1982. A selective medium for isolating *Campylobacter jejuni/coli*. J. Clin. Pathol. 35:462-467.

Bopp, C. A., J. G. Wells, and T. J. Barrett. 1982. Trimethoprim activity in media selective for *Campylobacter jejuni*. J. Clin. Microbiol. 16:808-812.

Butzler, J. P., P. Dekeyser, M. Detrain, and F. Dehaen. 1973. Related vibrio in stools. J. Pediatr. 82:493-495.

Butzler, J. P., and M. B. Skirrow. 1979. Campylobacter enteritis. Clin. Gastroenterol. 8:737-765.

Dekeyser P., M. Gossuin-Detrain, J. P. Butzler, and J. Sternon. 1972. Acute enteritis due to related vibrio: First positive stool cultures. J. Infect. Dis. 125:390-392.

Gavan, T. L., and A. L. Barry. 1980. Microdilution test procedure, p. 459-462. *In* E. H. Lennette, R. Balows, W. J. Hausler, and J. P. Traunt (ed.), Manual of clinical microbiology, 3rd ed. American Society of Microbiology, Washington, D.C.

Gebhart, C. J., G. E. Ward, and H. J. Kurtz. 1985. In vitro activities of 47 antimicrobial agents against three *Campylobacter* spp. from pigs. Antimicrob. Agents

Chemother. 27:55-59.

Goossens, H., M. De Boeck, and J. P. Butzler. 1983. A new selective medium for the isolation of *Campylobacter jejuni* from human faeces. Eur. J. Clin. Microbiol. 2:389-394.

Karmali, M. A., S. DeGrandis, and P. C. Fleming. 1980. Antimicrobial susceptibility of *Campylobacter jejuni* and *Campylobacter fetus* subsp. *fetus* to eight cephalosporins with special reference to species differentiation. Antimicrob. Agents Chemother. 18:948-951.

Karmali, M. A., S. DeGrandis, and P. C. Fleming. 1981. Antimicrobial susceptibility of *Campylobacter jejuni* with special reference to resistance patterns of Canadian isolates. Antimicrob. Agents Chemother. 19:593-597.

Karmali, M. A., and P. C. Fleming. 1979. Campylobacter enteritis in children. J. Pediatr. 94:527-533.

Karmali, M. A., and M. B. Skirrow. 1984. Taxonomy of the genus *Campylobacter*, p. 1-20. *In* J. P. Butzler (ed.), Campylobacter infection in man and animals. CRC Press Inc., Boca Raton, Fla.

Kucers, A., and N. McK. Bennett. 1975. The use of antibiotics, 2nd ed., p. 332-347. William Heinemann Medical Books Ltd., London.

Lauwers, S., M. De Boeck, and J. P. Butzler. 1978. Campylobacter enteritis in Brussels. Lancet i:604-605.

Lovett, J., D. W. Francis, and J. M. Hunt. 1983. Isolation of *Campylobacter jejuni* from raw milk. Appl. Environ. Microbiol. 46:459-462.

Michel, J., M. Rogol, and D. Dickman. 1983. Susceptibility of clinical isolates of *Campylobacter jejuni* to sixteen antimicrobial agents. Antimicrob. Agents Chemother. 23:796-797.

Ng, L.-K., M. E. Stiles, and D. E. Taylor. 1985. Comparison of basal media for culturing *Campylobacter jejuni* and *Campylobacter coli*. J. Clin. Microbiol. 21:226-230.

Ringertz, S., R. C. Rockhill, O. Ringertz, and A. Sutomo. 1981. Susceptibility of *Campylobacter fetus* subsp. *jejuni*, isolated from patients in Jakarta, Indonesia to ten antimicrobial agents. J. Antimicrob.

Chemother. 8:333-336.

Rosef, O., B. Gondrosen, G. Kapperud, and B. Underdal. 1983. Isolation and characterization of *Campylobacter jejuni* and *Campylobacter coli* from domestic and wild mammals in Norway. Appl. Environ. Microbiol. 46:855-859.

Saku, K., K. Sugimoto and Y. Uchyiama. 1983. Studies on susceptibility test for *Campylobacter jejuni/coli*. Jpn. J. Antibiot. 36:2757-2762.

Skirrow, M. B. 1977. Campylobacter enteritis: a "new" disease. Br. Med. J. 2:9-11.

Steers, E., E. L. Foltz, and B. S. Graves. 1959. Inocula replicating apparatus for routine testing of bacterial susceptibility to antibiotics. Antibiot. Chemother. 9:307-311.

Stern, N. J. 1982. Selectivity and sensitivity of three media for recovery of inoculated *Campylobacter fetus* ssp. *jejuni* from ground beef. J. Food Safety. 4:169-175.

Vanhoof, R. 1984. Susceptibility of campylobacters to antimicrobial agents, p.77-85. *In* J.-P. Butzler (ed.), Campylobacter infection in man and animals. CRC Press, Inc., Boca Raton, Fla.

Vanhoof, R., M. P. Vanderlinden, R. Dierickx, S. Lauwers, E. Yourassowsky, and J. P. Butzler. 1978. Susceptibility of *Campylobacter fetus* subsp. *jejuni* to twenty-nine antimicrobial agents. Antimicrob. Agents Chemother. 14:553-556.

Walder, M. 1979. Susceptibility of *Campylobacter fetus* subsp. *jejuni* to twenty antimicrobial agents. Antimicrob. Agents Chemother. 16:37-39.

Washington, J. A., and V. L. Sutter. 1980. Dilution susceptibility test: agar and macro-broth dilution procedures, p. 453-458. *In* E. H. Lennette, A. Balows, W. J. Hausler, and J. P. Truant (ed.), Manual of clinical microbiology, 3rd ed. American Society of Microbiology, Washington, D.C.

Waterworth, P. M. 1978. Quantitative methods for bacterial sensitivity testing, p. 31-40. *In* D. S. Reeves, I. Phillips, J. D. Williams, and R. Wise (ed.), Laboratory methods in antimicrobial chemotherapy. Churchill Livingstone, Edinburgh.

Wesley, R. D., B. Swaminathan, and W. J. Stadelman. 1983. Isolation and enumeration of *Campylobacter jejuni* from poultry products by a selective enrichment method. Appl. Environ. Microbiol. 46:1097-1102.

Whiffen, A. J. 1948. The production, assay, and antibiotic activity of actidione, an antibiotic from *Streptomyces griseus*. J. Bacteriol. 56:283-291.

Williams, J. D., and T. Leung. 1978. Polymyxins, rifamycins, nalidixic acid and nitrofurantoin, p. 88-93. *In* D. S. Reeves, I. Phillips, J. D. Williams, and R. Wise (ed.), Laboratory methods in antimicrobial chemotherapy. Churchill Livingstone, Edinburgh.

6. Isolation and Characterization of Animal Strains of *Campylobacter* species

6.1 Introduction

A bacterial culture originally derived from a single cell produces variants during prolonged subculturing on laboratory media. Among these changes are alterations in drug resistance patterns. It has been widely documented that most of these changes are due to the loss of drug resistance plasmids (R-factors) which occurs when the selective pressure is removed from the culture medium (Davies and Smith, 1978). Spontaneous chromosomal mutation to antibiotic resistance has also been observed. Several mechanisms of mutational resistance have been determined, including: (a) alteration of the target site of the antibiotic (Benveniste and Davies, 1973; Brakier-Gingras, 1974; Sinotnak, 1976); (b) alteration of permeability to the antibiotic (Sparling *et al.*, 1975; Gilleland *et al.*, 1984; Moore *et al.*, 1984); (c) increased production of antibiotic inactivating enzymes (Sykes and Matthew, 1976); and (d) increased production of a metabolite which competes with the antibiotic for the target site (Landy *et al.*, 1943).

Mutational changes in *C. jejuni* and *C. coli* that resulted in a change from nalidixic acid-susceptible to resistant strains have been observed (Taylor *et al.*, 1985). This phenomenon has also been observed for other DNA gyrase inhibitors, such as enoxacin (Taylor *et al.*, 1985). Based on such examples, it is reasonable to assume that the antibiotic susceptibilities of laboratory strains of bacteria may differ from fresh isolates.

The strains included in the earlier study of the inhibitory effect of antibiotics used in selective media (Ng *et al.*, 1985 described in Chapter 5) were laboratory strains that had been subcultured on synthetic laboratory media for unknown periods of time. Although some strains of *C. coli* were more susceptible than *C. jejuni* to antibiotics used in selective media, this could be a laboratory phenomenon and not typical of fresh isolates. In this study, fresh isolates of *C. jejuni* and *C. coli* from colons of cattle and swine were isolated by two methods: an antibiotic-containing medium and a filtration technique that does not use antibiotics in the selective process. The differentiation of *C. jejuni* and *C. coli* is equivocal, relying solely on the hippurate hydrolysis test. The fresh isolates were identified based on their phenotypic characteristics and, if necessary, by DNA homology determination. The antibiotic susceptibilities of the isolates, especially to antibiotics used in selective media, is described separately (see Chapter 8).

6.2 Materials and methods

6.2.1 Reference cultures

The reference strains of *Campylobacter* used for identification, biotyping and DNA homology studies included: *C. coli* NCTC 11353 (UA578), *C. jejuni* NCTC 11392 (UA579), *C. jejuni* NCTC 11168 (UA580), *C. fetus* subsp. *fetus* ATCC 27374 (UA60), *C. laridis* NCTC 11352 (UA577), *C. hyointestinalis* (UA564, supplied by J. H. Bryner, National Animal Disease Center, Ames, Iowa, U.S.A.), and *C. pyloridis* (supplied by H. Lior, Laboratory Centre for Disease Control, Ottawa, Canada).

6.2.2 Sources and isolation of *Campylobacter* spp.

Sections of the spiral colon of cattle and swine were obtained from the Provincial Veterinary Laboratory, Edmonton, Alberta; a local slaughter house; and the Agriculture Canada Research Station at Lacombe, Alberta. The animals raised at the Lacombe Research Station had a known antibiotic history. Trimethoprim had been used to treat cases of scours during weaning, otherwise antibiotics had not been used for therapeutic or growth purposes.

Approximately eighteen inches of the spiral colon was taken from the intestine of cattle and swine at the time of slaughter. In the laboratory, the sections of colon were cut open aseptically, and the gut contents removed without scraping the inner lining. Approximately six inches of the semi-cleaned colon was placed in a plastic bag with 50 mL of sterile 0.85% saline, and mixed in a Stomacher blender (Model 400, Colworth, London, England).

Procedures used for isolation of *Campylobacter* spp. included: (a) streaking unfiltered suspensions of the colon lining (prepared in the Stomacher blender, as described above) onto Mueller-Hinton (MH) (Oxoid, Basingstoke, England) agar and MH agar with polymyxin (2,500 IU/L), trimethoprim (5 mg/L) and vancomycin (10 mg/L) (SK) (Skirrow, 1972); (b) streaking the suspension filtrate from a Millipore filter of 0.65 µm pore size (Millipore Corp., Mississauga, Ontario) onto MH and SK media; and (c) enrichment of 1 mL of unfiltered suspension or the filtrate in 10 mL of brucella broth (Difco Laboratories, Detroit, MN, U.S.A.). Skirrow's combination of antibiotics was selected because it had previously been shown to be the least inhibitory to strains of *C. coli* and *C. jejuni* (Ng *et al.*, 1985).

Plates were examined after 24 and 48 h of incubation and typical *Campylobacter* colonies were selected for examination under phase contrast microscopy. Colonies with small spiral-shaped cells demonstrating a darting motility were streaked onto MH agar to obtain isolated colonies. Isolated colonies were selected and stored at -70°C in a sterile preservation medium containing glycerol (40%) and trisodium citrate (3%). If *Campylobacter* colonies were not detected on the solid media after 48 h of incubation, the brucella broth enrichments were examined for the presence of spiral-shaped cells, and streaked onto MH agar if appropriate.

6.2.3 Selection of *C. coli* and *C. jejuni* isolates

Typical *Campylobacter* colonies were randomly selected from the plates. Usually more than one colony and up to 25 colonies was selected from MH plates and one colony was selected from SK plates. The isolates were transferred aseptically to MH plates containing one of the following antibiotics: tetracycline (8 µg/mL), cephalothin (64 µg/mL) and kanamycin (8 µg/mL) and also onto MH containing the antibiotic combinations proposed by Skirrow (1977), Wesley *et al.* (1983), Dekeyser *et al.* (1972) and Goossens *et al.* (1983). The latter four antibiotic combinations were chosen based on differences in inhibition of *C. jejuni* and *C. coli* observed in an earlier study by Ng *et al.* (1985). From the different antibiotic plates, a representative group of *Campylobacter* cultures was selected which differed in antibiotic susceptibility.

6.2.4 Identification and biotyping of isolates

The isolates from cattle and swine were screened for the ability to produce oxidase and catalase and were identified using the tests listed in Table 6.1. The tests were done according to the descriptions of Morris and Patton (1986) except the for the susceptibility to nalidixic acid

Table 6.1 Differential characteristics of catalase positive *Campylobacter* species.

	Species[a]										
Tests[b]	Cj	Cc	Cl	Cff	Cfv	Cf	Ch	Cci	Cfe	Cp	AC
Growth											
25°C	-	-	-	+	+	-	V	-	-	ND	+
37°C	+	+	+	+	+	+	+	+	+	+	+
42°C	+	+	+	V	-	+	V	-	-	-	-
1% glycine	+	+	+	+	-	+	+	+	+	ND	V
3.5% NaCl	-	-	-	-	-	+	-	-	-	-	-
0.04% TTC	+	+	-	-	-	-	-	ND	ND	ND	ND
Biochemical											
nitrate reduction	+	+	+	+	+	+	+	+	-	-	-
H_2S in TSI	-	-	-	-	-	+	+	-	-	ND	-
alkaline phosphatase	V	V	ND	-	-	ND	ND	ND	ND	+	ND
urease	-	-	-	-	-	-	-	-	-	+	-
Others											
Nalidixic acid	S	S	R	R	V	V	R	S	S	R	S
Cephalothin	R	R	R	S	S	S	R	S	S	ND	R

---

[a] Abbreviations for *Campylobacter* species: Cj, *C. jejuni*; Cc, *C. coli*; Cl, *C. laridis*; Cff, *C. fetus* subsp. *fetus*; Cfv, *C. fetus* subsp. *venerealis*; Cfc, *C. faecalis*; Ch, *C. hyointestinalis*; Cci, *C. cinaedi*; Cfe, *C. fennelliae*; Cp, *C. pyloridis*; and AC, Aerotolerant *Campylobacter*

[b] V = variable; ND = not determined

(40 µg/mL) and cephalothin (64 µg/mL) in MH agar (Karmali and Skirrow, 1984). In addition the tolerance to 2,3,5-triphenyltetrazolium chloride (TTC) (400 µg/mL) in nutrient agar (Leaper and Owen, 1981), and the alkaline phosphatase test (Lewis, 1961) were included.

C. jejuni and *C. coli* isolates were also differentiated using the biotyping system proposed by Lior (1984), including DNA hydrolysis, and H_2S production in "FBP" medium, a semi-solid brucella broth containing 0.23% agar and 0.025% of each of ferrous sulfate, sodium metabisulfite and sodium pyruvate (see Table 6.2).

6.2.5 Total genomic DNA isolation and preparation

Campylobacter strains were inoculated onto ten MH agar plates (100 mm) to obtain confluent growth at 37°C for 24 or 48 h in an atmosphere containing 7% CO_2. Cells were harvested from the plates with 25 mL of saline-EDTA (0.15 M NaCl in 0.1 M EDTA, pH 8). The DNA was isolated from the cell suspension using the method described by Marmur (1961). The purity of the DNA was determined by absorbance readings at 260 nm and 280 nm in a spectrophotometer (DU-8, Beckman Instruments, Inc., Fullerton, CA, U.S.A.). DNA was purified with RNase (bovine pancrease; Calbiochem, La Jolla, CA, U.S.A.) and pronase (Boehringer Mannheim, Dorval, Quebec) to obtain A_{260}:A_{280} ratios of at least 1.8, as recommended by Marmur (1961). The isolated DNA were used for the determination of guanine and cytosine (G+C) content and

Table 6.2 Biotyping scheme for *C. jejuni*, *C. coli*, and *C. laridis*[1].

	C. jejuni				*C. coli*		*C. laridis*	
	I	II	III	IV	I	II	I	II
DNA hydrolysis	-	+	-	+	-	+	-	+
H_2S in FBP	-	-	+	+	-	-	+	+

[1] Lior, H. 1984. J. Clin. Microbiol. 20:636-640.

DNA-DNA homology studies. The DNA was dissolved in 0.1X SSC (1X SSC contains 0.15 M NaCl in 0.015 M sodium citrate, pH 7.0) to contain 1 to 2 mg of DNA/mL.

6.2.6 G+C determination

The base composition of the isolates was determined by measuring the Tm, which is defined as the mid-point of the thermal denaturation curve of DNA (Marmur and Doty, 1962). The Tm was determined by measuring the A_{260} of DNA in 0.1X SSC with a temperature programmed spectrophotometer (DU-8, Beckman) over a temperature range from 50° to 90°C. DNA from *Micrococcus lysodeikticus* (Sigma Chemical Co., St. Louis, MO, U.S.A.) with a known G+C content of 72 mol% (Marmur and Doty, 1962) and a Tm in 0.1X SSC of 85°C (Mandel *et al.*, 1970) was used as the standard. The relationship of base composition to Tm was determined according to Schildkraut and Lifson (1965) using the following equation:

$$Tm = 16.6 \times \log M + 0.41(G+C) + 81.5$$

where G+C is the mol% of guanine plus cytosine and M is the concentration of Na^+ in solution;

and according to the equation proposed by Mandel *et al.* (1970):

$$G+C = [(Tm_{0.1X\ SSC}/50.2)-.99] \times 100$$

where, $Tm_{0.1X\ SSC}$ is the Tm of DNA dissolved in 0.1X SSC.

6.2.7 DNA homology determination by slot blot hybridization

Slot blot hybridization was performed using the method proposed by Kafatos *et al.* (1979). The concentration of total genomic DNA in 0.1X SSC was adjusted to 20 µg/mL by measuring A_{260} in a spectrophotometer (DU-8, Beckman). The DNA was denatured for 15 min in 0.3 M NaOH at room temperature. Serial five-fold dilutions of denatured DNA were prepared in 1 M ammonium acetate (Kafatos, 1979; Boivin *et al.*, 1985). A nitrocellulose membrane filter (BA85, Schleicher and Schuell, Inc., Keene, NH, U.S.A.) was equilibrated by soaking in 1 M ammonium acetate for 20 min. Samples of diluted DNA (50 µL) were applied to the membrane filter using a slot blot manifold apparatus (Hybri-slot, Gibco/BRL Pacific, Ltd., Burlington, Ontario) to give 0.08 to 250 pg DNA per slot. The nitrocellulose sheet was soaked for 10 min in 4X SSC to remove dust particles and then air dried for 30 min. at room temperature. The DNA was fixed onto the membrane by baking at 65°C for 16 h.

The hybridization of the immobilized DNA on filters using DNA probes was performed as described by Portnoy *et al.* (1981). The filter was incubated at 42°C, in a heat sealable plastic bag (Philips, Scarborough, Ontario) containing 5 mL of pre-hybridization solution (250 µg herring sperm DNA; 50% formamide; 0.1% sodium dodecyl sulfate (SDS); 1 mM EDTA and 1X Denhardt's solution). 1X Denhardt's solution contains 0.02% polyvinyl-pyrrolidone (Sigma), 0.02% Ficoll 400 (Sigma) and 0.02% bovine serum albumin (Boehringer Mannheim, Dorval, Quebec). After 3 to 4 h of incubation the plastic bag was cut open and the pre-hybridization solution was drained out and 2.5 mL of hybridization solution was added to the bag. The hybridization solution was prepared by adding 250 μg of denatured herring sperm DNA, and denatured radioactive DNA probe to 2.5 mL of pre-hybridization solution. The sperm DNA and DNA probe was denatured by boiling at 100°C for 10 min. For each blot, 2 to 5 x 10^6 cpm of ^{32}P-labelled DNA probe was used. Hybridization was done at 42°C for 16 h. After hybridization the filter was washed for 1 h at 65°C with a solution containing 5X SSC, 0.1% SDS and 1 mM EDTA, followed by 2 washes (2 min each) at room temperature with 2X SSC. The filter was air dried and exposed to X-ray film (Kodak Xar, Eastman Kodak Co., Rochester, NY., U.S.A.) for 8 to 16 h at -70°C.

The degree of homology was determined by:
(a) visual examination of the intensity of the slots with different concentrations of DNA on the autoradiograms for heterologous and homologous DNA hybridization; and (b) cutting out the nitrocellulose filter strips containing individual slots and determining the radioactivity in a scintilation counter (LS6800, Beckman). The percentage DNA homology was calculated using the following equation (Seldin and Dubnau, 1985):

$$\%\ \text{homology} = \frac{\text{cpm of heterologous DNA}}{\text{cpm of homologous DNA}} \times 100$$

6.2.8 Preparation of ^{32}P-labelled DNA

The total genomic DNA from *Campylobacter* reference strains was labelled *in vitro* with [α-^{32}P]dCTP (New England Nuclear Corp., Boston, MA, U.S.A.) by a nick translation method described by Maniatis *et al.* (1975). The labelled DNA had specific activities of 10^7 to 10^8 cpm/μg of DNA.

6.2.9 Plasmid analysis

Selected *Campylobacter* strains were screened for plasmid DNA using a method based on that described by Birnboim and Doly (1979). The crude plasmid samples were analyzed by electrophoresis in 0.7% agarose at 60V for 5 h in Tris-borate-EDTA buffer (90 mM Tris, 90 mM boric acid, 25 mM Na_2 EDTA, pH 8.3) or at 35V in 0.7% agarose in Tris-acetate buffer (40 mM Tris-HCl, 20 mM sodium acetate, 2 mM EDTA, pH 8.0). *C. jejuni* UA466 which contains a 45 kb plasmid (Taylor, 1986) and its plasmid-free derivative (UA650) were used as positive and negative controls, respectively. *E. coli* plasmids of known molecular size (in kilobases, kb) were used as standards, including: pUC8, 2.7; pBR322, 4.0; RSF1030, 8.5; S-a, 35.4; R4776, 50.8; RP4, 58.5; RIP69, 72.4; LT_2, 92.4; RA-1, 132.4; R40-a, 147.8; R27, 172.5; Rts-1, 194.0; R478, 255.6. Gels were stained with ethidium bromide (0.4 μg/mL for 15 min) and DNA bands were visualized on a UV light box at 256 nm.

6.3 Results

A total of 92 colons (40 from cattle and 52 from swine) was obtained for isolation of *Campylobacter* spp. Most of the colons from cattle (38 out of 40) were from animals of known antibiotic history. Of the 52 colons from swine, 24 were of known antibiotic history. A summary of the frequency of isolation of *Campylobacter* strains is shown in Table 6.3.

Campylobacter strains were isolated from 65 (71%) of the 92 colons analyzed. Of the 65 colons yielding *Campylobacter* strains, 17 (26%) were from cattle and 48 (74%) were from swine, giving an isolation rate of 43% and 92%, respectively. The isolation rate from the bovine colons using the filtration method was 14 out of 40 (35%) and without filtration it was 12 out of 40 (30%). The isolation rate from the porcine colons using the filtration method was 44 out of 52 (85%) and without filtration was 40 out of 52 (77%). A total of 108 isolates was obtained from the filtration method, of which 40 were from cattle and 68 from swine. Using selective media (SK), a total of 67 isolates was selected, 19 from cattle and 48 from swine. The isolates were designated by colon number, and with an (a) if they were isolated using MH agar or a (b) if they were from MH agar with antibiotics (SK).

6.3.1 Identification and biotyping of isolates

The isolates were identified according to the phenotypic characteristics listed in Table 6.1. The results

Table 6.3 Frequency of isolation of *Campylobacter* strains from colons of 40 cattle and 52 swine.

Isolation technique[a]	no. (%) cattle positive colons	no. (%) swine positive colons
Filtrate		
MH[b]	9 (22.5)	40 (76.9)
MH + Skirrow's antibiotics	13 (32.5)	20 (38.5)
Brucella broth	9 (22.5)	29 (61.7)
Unfiltered		
MH	3 (7.5)	13 (25.0)
MH + Skirrow's antibiotics	11 (27.5)	36 (69.2)
Brucella broth	2 (5.0)	0 (0.0)

[a] The suspensions of colon samples were inoculated onto MH agar, MH agar with Skirrow's antibiotic combination (SK) and brucella broth before and after filtration through a 0,65 μm pore size membrane.

[b] MH : Mueller-Hinton agar.

are summarized in Table 6.4. The majority of the isolates from cattle colons were *C. jejuni* biotype I, only 6 isolates were biotype II. Similarly, 95% of isolates from swine colons were *C. coli* biotype I, and only 5% were biotype II. Only one *C. jejuni* isolate was obtained from swine, and *C. coli* was not isolated from cattle.

All isolates from cattle and swine were oxidase and catalase positive; able to grow at 37 and 42°C. Only 3 isolates from swine grew at 25°C. One of these isolates grew better at 37° than at 42°C and possessed biochemical characteristics which made it most probably a strain of *C. fetus* subsp. *venerealis*. The other two isolates that grew at 25°C had phenotypic characteristics similar to *C. coli*. They did not correspond with any of the identified *Campylobacter* spp., and they were probably atypical *C. coli*.

A total of 10 nalidixic acid resistant (Nal^R) strains was isolated. Eight of the Nal^R strains could not be characterized at the species level, based on their phenotypic characteristics. Some isolates were susceptible to 16 to 64 µg cephalothin per mL, including 5 isolates from cattle and 10 from swine. Eight of the cephalothin-susceptible strains were also susceptible to nalidixic acid and, based on other phenotypic characteristics, they were identified as *C. coli* biotype I or *C. jejuni* biotype II.

Based on the phenotypic characteristics of the isolates, it appeared that isolates of different phenotypes were isolated from the same colon, for example, *C. jejuni*

Table 6.4 The identification and classification of *Campylobacter* isolates from cattle and swine.

	Number of isolates	
Species	Cattle	Swine
C. jejuni		
biotype I	53	0
biotype II	6	1
C. coli		
biotype I	0	95
biotype II	0	5
C. coli/C. jejuni[1]	0	1
C. fetus subsp. *venerealis*	0	1
not identifiable	13	0

[1] Based on phenotypic characteristics this is either a *C. coli* or *C. jejuni*, but the hippurate hydrolysis tests gave an intermediate reaction.

biotypes I and II were obtained from one cattle colon. Different *Campylobacter* spp. were also isolated from the same colon, for example, one swine colon contained typical and atypical *C. coli* as well as other unidentifiable *Campylobacter* strains.

A total of 27 tetracycline-resistant isolates was isolated from 12 colons. Both cattle and swine yielded tetracycline-resistant strains. The tetracycline-resistant isolates will be discussed in more detail in Chapter 9. High level kanamycin-resistant strains were not encountered in this study, however, the Minimal Inhibition Concentrations (MICs) for kanamycin of some *C. coli* strains were 16 to 32 µg/mL.

6.3.2 Genetic characterization of cultures

To confirm the identification of some of the ~~animal~~ isolates, the G+C content of the total genomic DNA of selected isolates was determined, followed by DNA-DNA homology studies.

A. G+C determination

The G+C content of selected isolates was calculated from Tm values using the Schildkraut and Lifson (1965) and Mandel *et al.* (1970) equations. The results are shown in Table 6.5. The results calculated using the Schildkraut and Lifson (1965) equation were higher than those obtained using the Mandel *et al.* (1970) equation. However, the G+C values for the *Campylobacter* reference strains calculated by either

Table 6.5 The G+C composition of total genomic DNA of isolates from cattle and swine.

	G+C (mol%)[f]		
	(A)	(B)	Published data
Reference strains			
C. *coli* NCTC 11353 (UA578)	29.8	28.7	29-34
C. *jejuni* NCTC 11168 (UA580)	32.7	31.1	29-34
C. *fetus* subsp. fetus ATCC 27374 (UA60)	36.0	33.9	33-36
C. *laridis* NCTC 11352 (UA577)	31.5	30.0	32
C. *hyointestinalis* UA564	35.1	33.1	35-36
Fresh isolates[a]			
Typical strains			
C. *jejuni* I 6a	34.0	31.7	
C. *coli* I 4a	34.0	31.7	
C. *coli* I $25b_1$	32.0	30.5	
C. *coli* I 51b	32.0	30.5	
C. *coli* I 59a	35.1	33.1	
C. *coli* II 59b[b]	33.2	31.5	
Atypical strains			
C. *coli*/C. *jejuni* 1a[c]	32.0	30.5	
C. *coli* 2b[d]	32.0	30.5	
C. *coli* $51a_2$[e]	33.2	31.5	
unidentifiable $51a_1$	41.7	37.6	
unidentifiable $51a_3$	32.0	34.5	

[a] Classification based on phenotypic characteristics
[b] Tetracycline-resistant strain
[c] Hippurate hydrolysis intermediate
[d] Cephalothin-susceptible
[e] Growth at 25°C.
[f] G+C (mol%) was calculated from Tm values using equations: (A) Schildkraut and Lifson, 1965; and (B) Mandel *et al.*, 1970

method, except for *C. hyointestinalis*, were within the reported range of values (Karmali and Skirrow, 1984; Gebhart, 1985). The *C. hyointestinalis* strain used in this study had a lower G+C value than the strain (ATCC 35217) used by Gebhart (1985).

Typical and atypical fresh isolates which had been classified as *C. coli* and *C. jejuni* had G+C contents within the range reported in the literature (see Table 6.5). Atypical *C. jejuni* and *C. coli* strains included the cephalothin-susceptible strains, the strain with an intermediate hippurate hydrolysis reaction, and the strain that grew at 25°C. Isolates which could not be classified based on their phenotypic characteristics generally had higher G+C content (37 to 42 mol%) than *C. jejuni* and *C. coli*.

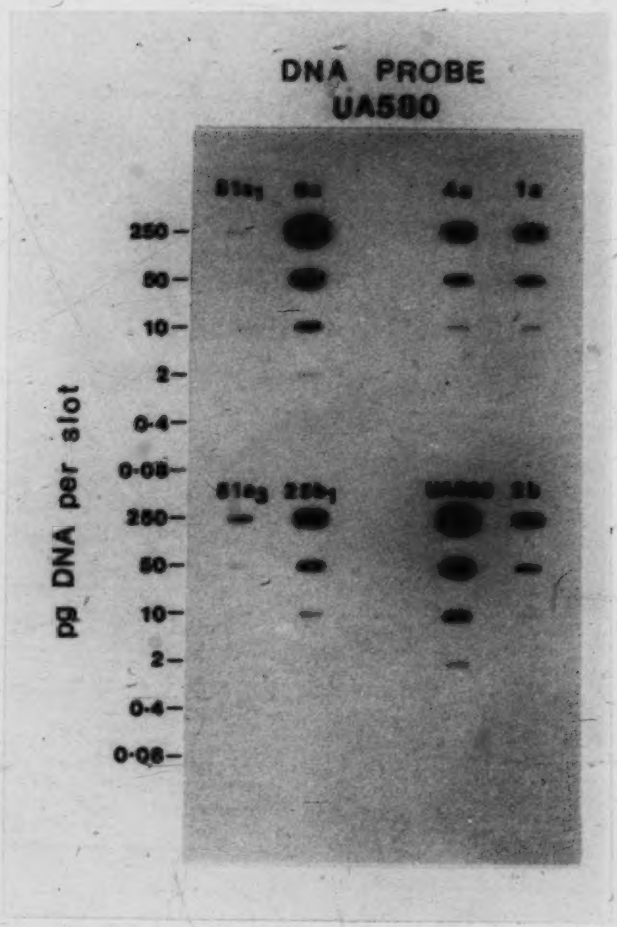
B. DNA homology studies

The extracted DNA for the G+C determination was also used for DNA homology studies using the slot blot hybridization technique. The DNA from twelve strains was hybridized with a ^{32}P-labelled DNA probe prepared from the total genomic DNA of *C. jejuni* NCTC 11168 (UA580). A representative sample of the autoradiograms obtained from the hybridizations is shown in Figure 6.1.

Visual examination of the intensity of the slots on the autoradiograms showed that of the isolates tested, only strain 6a had good homology with the reference *C. jejuni* probe; the *C. coli* fresh isolates 1a, 2b, 4a, and 25b$_1$

Figure 6.1 Autoradiogram prepared using the slot blot hybridization of DNA from *Campylobacter* isolates from cattle and swine with ^{32}P-labelled DNA probe prepared from the total genomic DNA of *C. jejuni* UA580.

The concentrations of the DNA (pg/slot) are indicated on the left margin.


DNA PROBE
UA580
pg DNA per slot
81a1
8a
4a
1a
250
50
10
2
0·4
0·08
81a3
28b1
UA580
2b
250
50
10
2
0·4
0·08

showed approximately 20% homology (about one dilution difference); while the unclassified strains 51a_1 and 51a_3 had little or no homology at the maximum concentration of DNA used. The amount of hybridization was measured by determining the level of radioactivity of the individual slots in a scintillation counter. Strain 6a had 100% homology with the *C. jejuni* probe, the *C. coli* strains had 11 to 40% homology, while the unclassified strains shared less than 5% homology.

The DNA from the isolates (except *C. jejuni* 6a) was also hybridized with a DNA probe prepared from the total genomic DNA of *C. coli* NCTC 11353 (UA578). The autoradiograms obtained from the hybridizations are shown in Figure 6.2. All of the typical and atypical *C. coli* strains, including strain 1a which had an intermediate hippurate reaction, had 74 to 88% homology with the *C. coli* DNA probe. The isolates with unknown identity showed <5% homology with the *C. coli* DNA probe.

When the three unclassifiable isolates (26a_2, 51a_1 and 51a_3) were hybridized separately with DNA probes prepared from the total genomic DNA of *C. laridis* NCTC 11352 (UA577), *C. hyointestinalis* (UA564) and *C. fetus* subsp. *fetus* (UA60), there was little or no homology with these species (see Figure 6.3). However, there was a high degree of homology among these isolates as illustrated by the hybridization of DNA from these isolates with a DNA probe prepared from the total genomic DNA of strain 51a_3 (see Figure 6.3, left

Figure 6.2 Autoradiogram of the slot blot hybridization of DNA from *Campylobacter* isolates with ^{32}P-labelled DNA probe from *C. coli* UA578.

The concentrations of DNA (pg/slot) are indicated on the left margin.

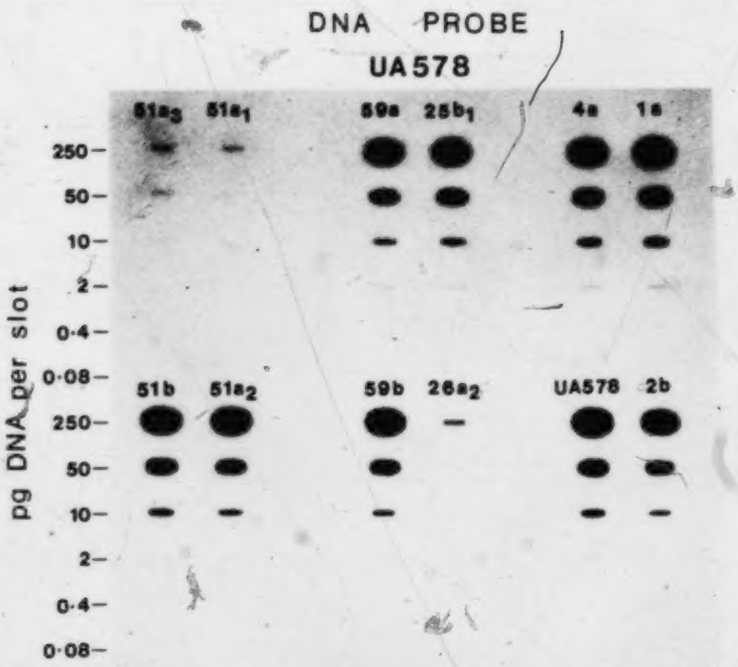
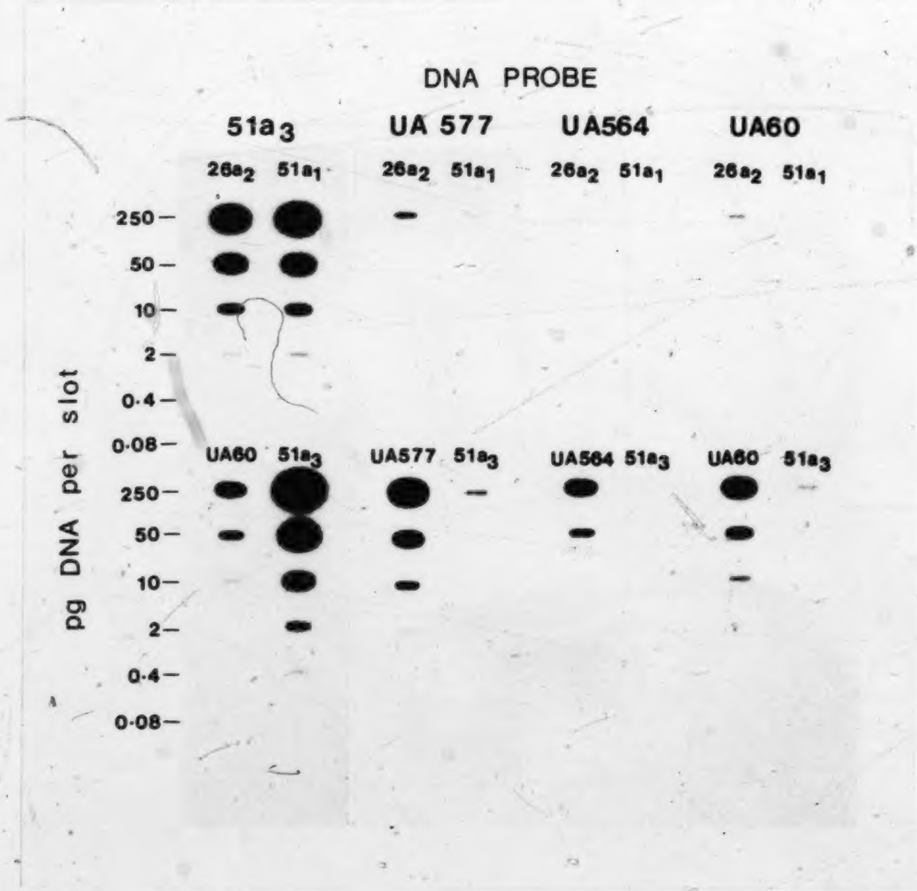

DNA PROBE
UA578
51a3
51a1
59a
25b1
4a
1a
250—
50—
10—
2—
0·4—
0·08—
pg DNA per slot
51b
51a2
59b
25a2
UA578
2b
250—
50—
10—
2—
0·4—
0·08—

Figure 6.3 The autoradiograms of DNA from unidentifiable isolates hybridized with DNA probes prepared from 51a$_3$, *C. laridis* UA577, *C. hyointestinalis* UA564, and *C. fetus* subsp. *fetus* UA60.

The concentrations of DNA (pg/slot) are indicated on the left margin.


DNA PROBE
51a3
UA 577
UA564
UA60
26a2
51a1
26a2
51a1
26a2
51a1
26a2
51a1
pg DNA per slot
250
50
10
2
0·4
0·08
UA60
51a3
UA577
51a3
UA564
51a3
UA60
51a3
250
50
10
2
0·4
0·08

panel).

6.3.3 Plasmid profiles

Plasmid profiles of selected *Campylobacter* strains were determined as an additional parameter for strain differentiation.

A. Isolates from cattle

A total of 25 isolates was selected for plasmid analysis. Of these isolates, ten were plasmid-free, the other 15 isolates, including the tetracycline-resistant strains, all carried one plasmid of approximately 87 kb. *C. jejuni* isolates from the same colon had the same plasmid profile. For example, the three *C. jejuni* isolates from colon 6 and the two *C. jejuni* isolates from colon 10 were plasmid-free, whereas, all of the tetracycline-resistant *C. jejuni* isolates from colon 9 carried the 87 kb plasmid.

B. Isolates from swine

Isolates from five porcine colons (24, 25, 26, 29 and 51) and a few randomly selected strains were analyzed for their plasmid contents. The results are summarized in Table 6.6. The plasmid profiles of *C. jejuni* $24a_1$ and *C. coli* $24b_1$ were the same, but *C. coli* $24b_2$ was missing the plasmid band of approximately 26 kb. The *C. coli* isolates from colon 25 contained only one plasmid, but the plasmid in $25b_1$ appeared to be slightly larger than the plasmid in other isolates. *C. coli* isolated from colon 26 had same plasmid profile, but the unidentifiable isolate $26a_2$ was plasmid-free. All of the

Table 6.6 The plasmid profiles of isolates from swine determined by gel electrophoresis.

Strains	Species	Estimated plasmid size (kb)
1a, 1b	*C. coli* I[a]	100, 8.7, 6.0, 2.9, 2.4, 2.0
2b	*C. coli* I	78, 9.5, 5.4, 3.2, 1.7
24a$_1$	*C. jejuni* II	148, 26, 5.2, 4.6, 3.1, 2.6
24b$_1$	*C. coli* I	148, 26, 5.2, 4.6, 3.1, 2.6
24b$_2$	*C. coli* I	148, 5.1, 4.6, 3.1
25a$_1$, 25a$_2$, 25a$_3$, 25a$_4$, 25b$_2$, 25b$_3$	*C. coli* I	106
25b$_1$	*C. coli* I[a]	155
26a$_1$, ~~26a$_4$, 26b~~	*C. coli*	7.1, 4.3, 4.0
26a$_2$	unknown	plasmid-free
28a$_1$	*C. coli* I[b]	54.7, 3.0, 2.4
29a$_1$, 29a$_2$, 29a$_3$, 29b$_1$	*C. coli* I[a]	123
29b$_2$	*C. coli* I	126, 72, 8.3, 4.1
51a$_1$, 51a$_3$	unknown	8.16, 4.5
51a$_2$	*C. coli*[c]	plasmid-free
51b$_1$	*C. coli* I	53, 25.7
75a$_1$	*C. coli* II[a]	93.3, 53.0, 10.3
75b	*C. coli* I	93.3, 53.0, 10.3
76a	*C. coli* I	169, 2.3

[a] Tetracycline-resistant
[b] Cephalothin-susceptible (MIC 32 to 64 µg/mL)
[c] Grows at 25°C

tetracycline-resistant strains from colon 29 had a plasmid of approximately 123 kb, but the tetracycline-susceptible isolate ($29b_2$) had a very different plasmid profile. There were also differences in plasmid profiles among the isolates from colon 51. The two strains of *C. coli* from colon 75, were of different biotypes, but they had the same plasmid profiles. The sizes of the plasmids depends on the electrophoresis conditions and will be further discussed in Chapter 9. In summary, *C. coli* cultures from different colon samples had different plasmid profiles. The *C. coli* cultures from the same colon may or may not have the same plasmid profile.

6.4 Discussion

6.4.1 Isolation of cultures

The surface of the spiral colon was used as the source of *Campylobacter* strains instead of feces. Earlier studies on the intestinal colonization of *Campylobacter* spp. (Field *et al.*, 1981; Lee *et al.*, 1986; Yrios and Balish, 1986) demonstrated that these organisms were closely associated with the epithelial cell brush border and the mucosal crypts and glands of the intestines of animals. This study showed that both the filtration method and the SK medium (MH agar with Skirrow's antibiotic combinations) were similarly efficient in isolating *Campylobacter* organisms. This is because SK medium is not inhibitory to *Campylobacter* strains

(Ng *et al*. 1985, Chapter 5). For the isolation of low numbers of *Campylobacter* in samples, it was shown that large volumes of filtrate from the filtration method can be inoculated into enrichment broth to increase the isolation rate. A less laborious modified filtration method described by Steele and McDermott (1984), in which the filter membrane is placed directly on non-selective agar. The limitation of the modified method was the small volume which could be applied on the membrane, therefore, this method was not included in this study.

C. coli and *C. jejuni* are usually considered to be resistant to cephalothin (64 µg/mL), and this is used for differention and selection purposes. In this study, 8 out of 161 *C. coli* and *C. jejuni* strains had MICs for cephalothin between 32 to 64 µg/mL, and showed inhibition by a 30 µg cephalothin disk. They were therefore, considered to be cephalothin-susceptible. Fresh isolates with very low MICs for cephalothin were not encountered in this study, although other workers (Brooks *et al*., 1986; Karmali and Skirrow, 1984; Mégraud and Elharrif, 1985) have isolated *Campylobacter* strains susceptible to colistin (50 µg disk) and cephalothin (4 to 16 µg/mL). Two *C. coli* strains, including the reference strain *C. coli* NCTC 11353 and a fresh isolate, became susceptible (MIC 4 µg/mL) after subculturing on MH agar. The other characteristics of these two variants that were tested remained unchanged. Therefore, the reliability of cepholothin resistance as differential and selection characteristics should be re-evaluated.

Plasmid-mediated kanamycin resistance has been reported in *C. coli* (Lambert *et al.*, 1985; Kotarski *et al.*, 1986) and *Campylobacter*-like organisms (Ouellette *et al.*, 1986). In this study, none of the isolates was resistant to high levels of (>16 µg/mL) kanamycin. In the Alberta Laboratory for Public Health, only one kanamycin-resistant *C. coli* strain was detected in the period from 1984 to 1986 (L. Mueller, personal communication). It appeared that kanamycin-resistance is not as prevalent in *Campylobacter* strains in Alberta as tetracycline-resistance, which agrees with the results of Taylor *et al.* (1986). Therefore, only tetracycline-resistance was useful as an antibiotic resistance marker for strain differentiation in this study.

6.4.2 Identification of isolates from animals

At present, there are only limited phenotypic characteristics available for the identification and taxonomic classification of *Campylobacter* species. The differentiation of *C. jejuni* and *C. coli* relies solely on the hippurate hydrolysis test. Atypical characteristics of some *Campylobacter* strains makes their classification difficult. In this study, an intermediate color reaction in the hippurate hydrolysis test was encountered for one strain, so it could not be classified as either *C. coli* or *C. jejuni*. Using DNA homology study, this culture was identified as a *C. coli* strain. Although no false positive hippurate hydrolysis strains have been reported in the literature, hippurate-negative *C. jejuni* strains have been encountered and their identity confirmed by DNA homology studies (Roop *et al.*, 1985; Totten *et al.*, 1985).

A semi-quantitative method was used to study the DNA homology of some isolates to confirm their taxonomic classification. Recently, semi-quantitative methods have been used to determine the taxonomic classification of other bacteria, *Aquaspirillium* spp. (Boivin *et al.*, 1985), *Bacillus* spp., (Seldin and Dubnau, 1985) and *Mycobacterium* spp. (Athwal *et al.*, 1984). The reliability of this semi-quantitative method was tested against a spectrophotometric DNA hybridization assay (Athwal *et al.*, 1984). This method is simpler to perform than either the S-1 nuclease (Crosa *et al.*, 1973) or the hydroxyapatite (Brenner *et al.*, 1969) methods. The slot blot method will be more advantageous than other methods for routine use, especially when non-radioactive probes become available for visualizing the "slots".

The G+C content of some of the unidentifiable isolates was relatively high (37 to 42 mol%) compared with the reference strains which had G+C contents of 30 to 36 mol%. Although an asparagine-fermenting *Campylobacter* spp. from an anaerobic digester was found to have an unusually high G+C content (41.6 mol%), the taxonomic position of the unidentifiable isolates in this study remain uncertain.

6.4.3 Biotyping of isolates

The Lior (1984) biotyping system which includes DNA hydrolysis and production of H_2S in FBP medium, was used to characterize *C. coli* and *C. jejuni* strains. The majority of the *C. coli* and *C. jejuni* strains belonged to biotype I. This is similar to findings in other reports (Karmali and Skirrow, 1984; Lior, 1984). None of the *C. coli* or *C. jejuni* isolates produced alkaline phosphatase, although alkaline phosphatase-positive strains have frequently been encountered in other studies (Smibert, 1974; Roop *et al.*, 1984). Therefore, the alkaline phosphatase test was not useful for differentiating *C. coli* and *C. jejuni* strains in this study. However, some of the unidentifiable isolates produced alkaline phosphatase, which made them distinguishable from other isolates from the same colon samples.

6.4.4 Plasmid profiles

The plasmid profiles of *C. jejuni* isolates in this study differed markedly from those for *C. coli*. *C. jejuni* biotypes I and II were either plasmidless or carried a plasmid of approximately the same size (87 kb). Therefore, plasmid profiles were not useful for strain differentiation among *C. jejuni* isolates in this study. On the other hand, isolates from the porcine colons had a wider spectrum of plasmid profiles, and many strains carried several plasmids. Therefore, plasmid profiles can be used to differentiate strains with the same biotype. Some strains of *C. coli* biotype I from the same colon showed different plasmid profiles, as a result for epidemiological purposes, it would be necessary to select more than one isolate from a single sample.

In summary, this study showed that both the filtration method and selective medium (with Skirrow's combination of antibiotics) were suitable for the isolation of *Campylobacter* strains from animal sources. Some of these isolates were cephalothin-susceptible and may not be isolated with selective media containing cephalothin. The effect of antibiotics used in selective media on some of these fresh isolates will be further studied (see Chapter 8). From this study it is apparent that biotype alone is not sufficient for strain differentiation, since the majority of the strains belong to biotype I. Antibiotic susceptibility may be a useful marker. Plasmid profiles alone may not be useful for epidemiological studies involving *C. jejuni*. On the other hand, the *C. coli* isolates had a much wider spectrum of plasmid profiles and many strains carried more than one plasmid. As a result, plasmid profiles may be a more useful parameter for differentiating strains of *C. coli*. Since several species or strains can be found in one animal, this may further complicate the identification of the source of clinical isolates in epidemiological studies.

6.5 Bibliography

Athwal, R. S., S. S. Deo, and T. Imaeda. 1984. Deoxyribonucleic acid relatedness among *Mycobacterium leprae*, *Mycobacterium lepraemurium*, and selected bacteria by dot blot and spectrophotometric deoxyribonucleic acid hybridization assays. Int. J. Syst. Bacteriol. 34:371-375.

Benveniste, R., and J. Davies. 1973. Mechanisms of antibiotic resistance in bacteria. Annu. Rev. Biochem. 42:471-506.

Brakier-Gingras, L. 1974. Resistance to antibiotics and alterations in the bacterial ribosome. Biomedicine. 20:267-272.

Brenner, D. J., G. R. Fanning, K. E. Johnson, R. V. Citarella, and S. Falkow. 1969. Polynucleotide sequence relationships among members of *Enterobacteriaceae*. J. Bacteriol. 98:637-650.

Brooks, B. W., M. M. Garcia, A. D. E. Fraser, H. Lior, R. B. Stewart and A. M. Lammerding. 1986. Isolation and characterization of cephalothin-susceptible *Campylobacter coli* from slaughter cattle. J. Clin. Microbiol. 24:591-595.

Boivin, M. F., V. L. Morris, E. C. M. Lee-Chan, and R. G. E. Murray. 1985. Deoxyribonucleic acid relatedness between selected members of the genus *Aquaspirillum* by slot blot hybridization: *Aquaspirillum serpens* (Mueller 1786) Hylemon, Wells, Krieg, and Jonnasch 1973 emended to include *Aquaspirillum bengal* as a subjective synonym. Int. J. Syst. Bacteriol. 35:512-517.

Crosa, J. H., D. J. Brenner, and S. Falkow. 1973. Use of a single-strand specific nuclease for analysis of bacterial and plasmid deoxyribonucleic acid homo- and heteroduplexes. J. Bacteriol. 115:904-911.

Davies, J., and D. I. Smith. 1978. Plasmid-determined resistance to antimicrobial agents. Annu. Rev. Microbiol. 32:469-518.

Dekeyser, P., M. Gossuin-Detrain, J. P. Butzler, and J. Sternon. 1972. Acute enteritis due to related vibrios: First positive stool cultures. J. Infect. Dis. 125:390-392.

Field, L. H., J. L. Underwood, L. M. Pope, and L. J. Berry. 1981. Intestinal colonization of neonatal animals by

Campylobacter fetus subsp. *jejuni*. Infect. Immun. 33:884-892.

Gebhart, C. J., P. Edmonds, G. E. Ward, H. J. Kurtz, and D. J. Brenner. 1985. "*Campylobacter hyointestinalis*" sp. nov.: a new species of *Campylobacter* found in the intestines of pigs and other animals. J. Clin. Microbiol. 21:715-720.

Gilleland, H. E., F. R. Champlin, and R. S. Conrad. 1984. Chemical alterations in cell envelopes of *Pseudomonas aeruginosa* upon exposure to polymyxin: a possible mechanism to explain adaptive resistance to polymyxin. Can. J. Microbiol. 30:869-873.

Goossens, H., M. De Boeck, and J. P. Butzler. 1983. A new selective medium for the isolation of *C. jejuni* from human faeces. Eur. J. Clin. Microbiol. 2:389-394.

Kafatos, F. C., C. W. Jones, and A. Efstratiadis. 1979. Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridization procedure. Nucleic Acids Res. 7:1541-1552.

Karmali, M. A., and M. B. Skirrow. 1984. Taxonomy of the genus *Campylobacter*, p. 1-20. *In* J. P. Butzler (ed.), Campylobacter infection in man and animals. CRC Press, Inc., Boca Raton, Fla.

Kotarski, S. F., T. L. Merriwether, G. T. Tkalcevic, and P. Gemski. 1986. Genetic studies of kanamycin resistance in *Campylobacter jejuni*. Antimicrob. Agents Chemother. 30:225-230.

Lambert, T., G. Gerbaud, P. Trieu-Cuot, and P. Courvalin. 1985. Structural relationship between the genes encoding 3'-aminoglycoside phosphotransferases in *Campylobacter* and in gram-positive cocci. Ann. Inst. Pasteur 136B:135-150.

Landy, M., N. W. Larkum, E. J. Oswald, and F. Streightoff. 1943. Increased synthesis of *p*-aminobenzoic acid associated with development of sulfonamide resistance in *Staphylococcus aureus*. Science 97:265-267.

Leaper, S., and R. J. Owen. 1981. Identification of catalase-producing *Campylobacter* species based on biochemical characteristics and on cellular fatty acid composition. Curr. Microbiol. 6:31-35.

Lee, A., J. L. O'Rourke, P. J. Barrington, and T. J. Trust. 1986. Mucous colonization as a determinant of pathogenicity in intestinal infection by

Campylobacter jejuni: A mouse cecal model. Infect. Immun. 51:536-546.

Lewis, B. 1961. Phosphatase production by Staphylococci - A comparison of two methods. J. Med. Lab. Technol. 18:112-113.

Lior, H. 1984. New, extended biotyping scheme for *Campylobacter jejuni*, *Campylobacter coli*, and "*Campylobacter laridis*". J. Clin. Microbiol. 20:636-640.

Mandel, M., L. Igambi, J. Bergendahl, M. L. Dodson, and E. Scheltgen. 1970. Correlation of melting temperature and cesium chloride buoyant density of bacterial deoxyribonucleic acid. J. Bacteriol. 101:333-338.

Maniatis, T., A. Jeffrey, and D. G. Kleid. 1975. Nucleotide sequence of the rightward operator of phage lambda. Proc. Natl. Acad. Sci. U.S.A. 72:1184-1188.

Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. 3:208-218.

Marmur, J., and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J. Mol. Biol. 5:109-118.

Mégraud, F., and Z. Elharif. 1985. Isolation of *Campylobacter* species by filtration. Eur. J. Clin. Microbiol. 4:437-438.

Moore, R. A., L. Chan, and R. E. W. Hancock. 1984. Evidence for two distinct mechanisms of resistance to polymyxin B in *Pseudomonas aeruginosa*. Antimicrob. Agent Chemother. 26:539-545.

Morris, G. K., and C. M. Patton. 1985. *Campylobacter*, p. 302-308. *In* E. H. Lennette, A. Balows, W. J. Hausler and H. J. Shadomy (ed.), Manual of clinical microbiology, 4th ed. American Society for Microbiology, Washington, D.C.

Ng, L.-K., M. E. Stiles, and D. E. Taylor. 1985. Inhibition of *Campylobacter coli* and *Campylobacter jejuni* by antibiotics used in selective growth media. J. Clin. Microbiol. 22:510-514.

Ouelette, M., G. Gerbeau, T. Lambert, and P. Courvalin. 1986. Cloning and sequencing of a 3'aminoglycoside phosphotransferase (APH3') found in *Campylobacter*-like organisms (CLO). Abstr. Annu.

Meet. Canad. Soc. Microbiol. GM9, p.79.

Portnoy, D. A., S. L. Moseley, and S. Falkow. 1981. Characterization of plasmids and plasmid-associated determinants of *Yersinia enterocolitica* pathogenesis. Infect. Immun. 31:775-782.

Roop, R. M., II, R. M. Smibert, and N. R. Krieg. 1984. Improved biotyping schemes for *Campylobacter jejuni* and *Campylobacter coli*. J. Clin. Microbiol. 20:990-992.

Schildkraut, C. and S. Lifson. 1965. Dependence of the melting temperature of DNA on salt concentration. Biopolymers 3:195-208.

Seldin, L., and D. Dubnau. 1985. Deoxyribonucleic acid homology among *Bacillus polymyxa*, *Bacillus macerans*, *Bacillus azotofixans* and other nitrogen-fixing *Bacillus* strains. Int. J. Syst. Bacteriol. 35:151-154.

Sirotnak, F. M. 1976. Chromosomal mutation to drug resistance in bacteria. Antibiot. Chemother. 20:67-86.

Skirrow, M. B. 1977. Campylobacter enteritis: a "new" disease. Br. Med. J. 2:9-11.

Sparling, P. F., F. A. Sarubbi, Jr., and E. Blackman. 1975. Inheritance of low-level resistance to penicillin, tetracycline, and chloramphenicol in *Neisseria gonorrhoeae*. J. Bacteriol. 124:740-749.

Steele, T. W., and S. N. McDermott. 1984. The use of membrane filters applied directly to the surface of agar plates for the isolation of *Campylobacter jejuni* from feces. Pathol. 16:263-265.

Sykes, R. B., and M. Matthew. 1976. The β-lactamases of gram-negative bacteria and their role in resistance to β-lactam antibiotics. J. Antimicrob. Chemother. 2:115-157.

Taylor, D. E. 1986. Plasmid-mediated tetracycline resistance in *Campylobacter jejuni*: Expression in *Escherichia coli* and Identification of homology with streptococcal class M determinant. J. Bacteriol. 165:1037-1039.

Taylor, D. E., N. Chang, R. S. Garner, R. Sherburne, and L. Mueller. 1986. Incidence of antibiotic resistance and characterization of plasmids in *Campylobacter jejuni* strains isolated from clinical sources in Alberta, Canada. Can. J. Microbiol. 32:28-32.

Taylor, D. E., H. Lior, and L.-K. Ng. 1985. Susceptibility of *Campylobacter* species to nalidixic acid, enoxacin, and other DNA gyrase inhibitors. Antimicrob. Agent Chemother. 28:708-710.

Totten, P. A., F. C. Tenover, K. C. S. Chen, K. L. Bruch, K. K. Holmes, and W. E. Stamm. 1985. Rapid genetic grouping test for identification of *Campylobacter* species, p. 234. *In* A. D. Pearson, M. B. Skirrow, H. Lior, and B. Rowe (ed.). Campylobacter III: Proceedings of the Third International Workshop on Campylobacter Infections, Ottawa. Public Health Laboratory Service, London.

Wesley, R. D., B. Swaminathan, and W. J. Stadelman. 1983. Isolation and enumeration of *Campylobacter jejuni* from poultry products by a selective enrichment method. Appl. Environ. Microbiol. 46:1097-1102

Yrios, J. W., and E. Balish. 1986. Colonization and infection of athymic and euthymic germfree mice by *Campylobacter jejuni* and *Campylobacter fetus* subsp. *fetus*. Infect. Immun. 53:378-383.

7. Determination of DNA homology among *Campylobacter* strains using chromosomal DNA probes

7.1 Introduction

Molecular genetic techniques have been applied in systematic bacteriology for the classification of bacteria. With information on base-ratio composition (G+C mol%) of DNA and DNA homology determinations, some bacteria which were previously grouped according to phenotypic data have been reclassified (Johnson, 1985). Members of the genus *Campylobacter* have guanine plus cytosine (G+C) content ranging from 29 to 38 mol% (Smibert, 1985). This enabled them to be distinguished from the genus *Vibrio* which has G+C ranging from 38 to 51 mol% (Baumann *et al.*, 1985). Furthermore, using DNA homology studies, *C. jejuni* and *C. coli* are now recognized as distinct species (Hébert *et al.*, 1984; Owen and Leaper, 1981); and in the current edition of *Bergey's Manual of Systematic Bacteriology* (Smibert, 1985) they are no longer considered as subspecies of *C. fetus*. Recently, Totten *et al.* (1985) used G+C and DNA homology determinations, in addition to biochemical tests, to distinguish *Campylobacter*-like organisms from other *Campylobacter* species. As a result, two new species, *C. cinaedi* and *C. fennelliae*, were proposed.

In the previous study (Chapter 6), some atypical *Campylobacter* strains were confirmed as *C. coli* or *C. jejuni* using the slot-blot hybridization method described by

Kafatos *et al.* (1979). This semi-quantitative method for the determination of DNA homology between bacteria requires the isolation of pure DNA. In this study, an attempt was made to modify the slot-blot technique by eliminating the necessity for DNA isolation. Whole cells were applied to nitrocellulose filter membranes for *in situ* hybridization.

7.2 Materials and methods

7.2.1 Bacterial strains

Reference strains were included from type culture collections: *C. coli* NCTC 11353 (UA578), *C. jejuni* biotype I NCTC 11168 (UA580), *C. jejuni* biotype II NCTC 11392 (UA579), *C. fetus* subsp. *fetus* ATCC 27374 (UA60). In addition, *C. laridis* UA487 (supplied by H. Lior, Laboratory Centre for Disease Control, Ottawa, Canada) and *C. coli* strain $29a_1$ isolated from a porcine colon in the previous study (Chapter 6) were used as test organisms. All cultures were maintained by subculturing weekly on Mueller-Hinton (MH) agar (Oxoid Ltd., Basingstoke, England). For dot blot hybridization, a 24-h culture in MH broth was used. All cultures were incubated at 37°C in an atmosphere of 7% CO_2.

7.2.2 Dot blot hybridization

Overnight cultures of *Campylobacter* strains grown in MH broth were adjusted with sterile MH broth to similar turbidity by measuring the A_{600} in a spectrophotometer

(DU-8, Beckman Instruments, Inc., Fullerton, CA, U.S.A.). Serial 4-fold dilutions of the cell suspensions were prepared and a 100 μL sample of each dilution was loaded onto a nitrocellulose membrane filter (0.2 μm, Schleicher and Schuell Inc., Keene, NH, U.S.A.) using a dot blot apparatus (Bio-Dot, Bio-Rad Laboratories, Richmond, CA, U.S.A.). The concentration of the cell suspensions (about 10^8 CFU/mL) was determined by plating onto MH agar and incubating at 37°C for 48 h. The cells on the nitrocellulose filter membrane were lysed and their DNA denatured by placing the nitrocellulose filter on a Whatman #3 filter paper saturated with 0.5 M NaOH for 10 min. After lysing the cells, the NaOH was neutralized by three sequential 1 min washings with 1 M Tris, pH 7.0, followed by a 10 min wash in 1.5 M NaCl in 1.0 M Tris, pH 7.0. The filter was treated with pronase (10 μg/mL) (Boehringer Mannheim, Dorval, Quebec) for 30 min; dried at room temperature for 15 min; and given two chloroform washes (1 min per wash). The prepared filter was air dried for 15 min and soaked in NaCl-Tris (1.5 M NaCl, 1.0 M Tris, pH 7.0) solution for 10 min. The DNA was fixed onto the filter by baking at 65°C overnight. The baked nitrocellulose membrane filter was placed in a heat-sealable plastic bag with 10 mL of pre-hybridization solution containing 50% formamide, 1% glycine, 50 mM sodium phosphate buffer at pH 6.5, 5X SSPE and 5X Denhardt's solution (1X SSPE contains 0.15 M NaCl, 10 mM NaH_2PO_4 and 1 mM Na_2 EDTA, pH 7.7; 1X Denhart's solution contains 0.02% polyvinyl-pyrrolidone, 0.02% Ficoll 400, and 0.02% bovine serum albumin). The filter was incubated at 42°C for 4 h.

The pre-hybridization solution was poured out and 2.5 mL of hybridization solution was added to the filter. The hybridization solution contained ^{32}P-labelled DNA probe (10^6 cpm/blot) prepared from total genomic DNA of *C. coli* UA578 or *C. jejuni* UA580, 100 µg/mL herring sperm DNA (Sigma Chemical Co., St. Louis, MO, U.S.A.), 50% formamide, 1% glycine, 20 mM sodium phosphate, 5X SSPE, 1X Denhardt's solution, and 10% dextran sulfate. The DNA probe and herring sperm DNA were denatured by heating in the hybridization solution at 100°C for 10 min before use. Hybridization was done at 37°C and 42°C for 16 h. At the end of the hybridization period, the nitrocellulose filter was washed three times at room temperature with 2X SSPE plus 0.1% SDS (5 min per wash) and once at 58°C for 30 min with 0.1X SSPE plus 0.1% SDS.

7.2.3 DNA-DNA homology determination

Autoradiograms of the air-dried filters were prepared by exposure to X-ray film (Kodak Xar, Eastman Kodak Co., Rochester, NY, U.S.A.) for 5 h to 2 days at -70°C. The autoradiograms were compared visually to determine the extent of hybridization. A serial four-fold dilution of each *Campylobacter* strain was spotted onto nitrocellulose filters. Therefore, one dilution difference in the intensity of the dots between the heterologous hybridization and the homologous hybridization represents 25% homology with the DNA probe.

7.2.4 Preparation of ^{32}P-labelled DNA.

The total genomic DNA from *C. coli* (UA578) and *C. jejuni* (UA580) reference strains was labelled *in vitro* with [α-^{32}P]dCTP (New England Nuclear Corp., Boston, MA, U.S.A.) by the nick translation method described by Maniatis *et al.* (1975). The labelled DNA had specific activities of 10^7 to 10^8 cpm/μg of DNA. The sonicated DNA from *C. coli* (UA578) was prepared shearing the DNA with an ultrasonic Dismembrator (Model 300, Artek Systems Corp., Farmingdale, NY, U.S.A.) for 10 min. The fragment sizes of the sonicated DNA were determined by electrophoresis in 5% polyacrylamide gel.

7.3 Results

Four strains of *Campylobacter* including *C. jejuni* UA580, *C. fetus* subsp. *fetus* UA60, *C. laridis* UA487, and *C. coli* UA578 were hybridized with a ^{32}P-labelled DNA probe prepared from the total genomic DNA isolated from *C. jejuni* (UA580). The results are illustrated in the autoradiograms shown in Figure 7.1. The results presented in the upper panel were hybridized at 42°C, and those in the lower panel were hybridized at 37°C, representing two levels of stringency conditions. The dots in column 1 were "spotted"

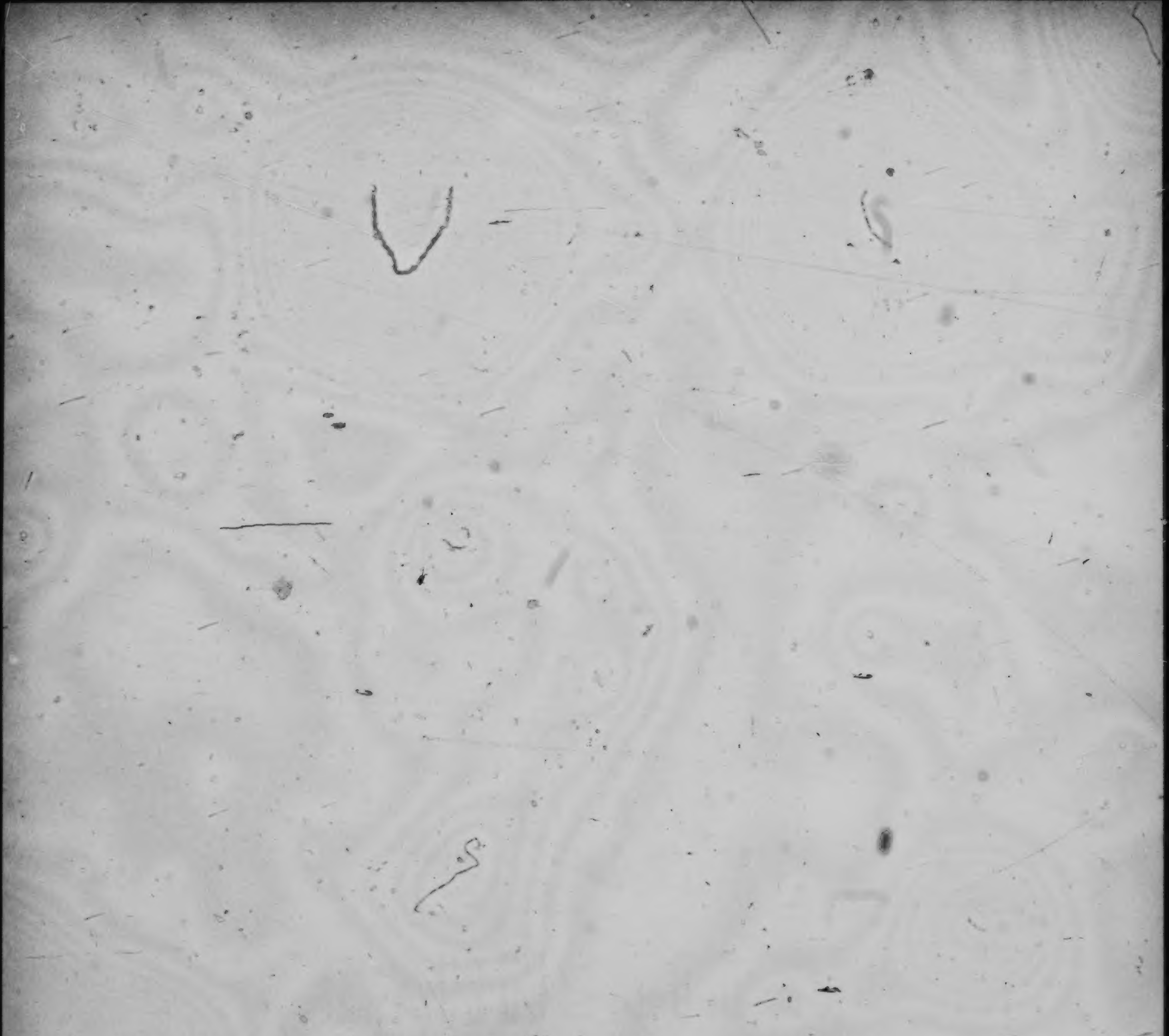
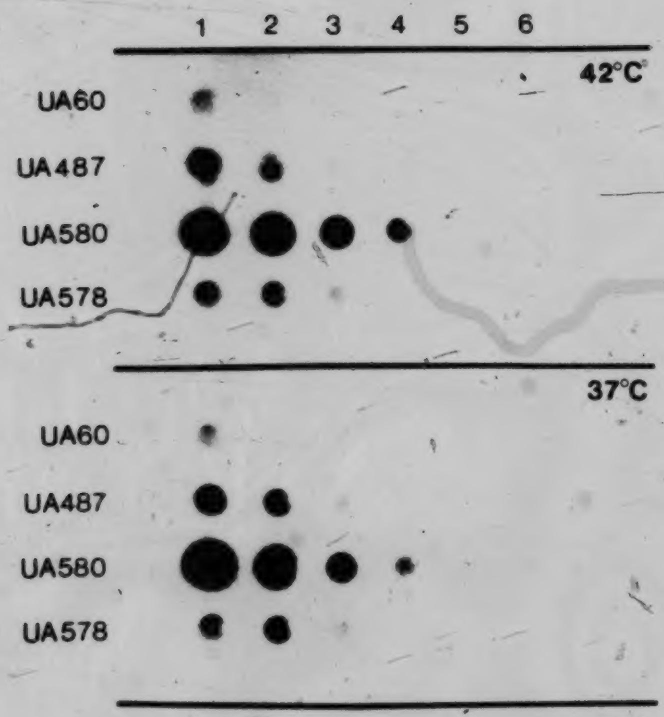


Figure 7.1 Autoradiogram of dot blot hybridization of *Campylobacter* strains with total genomic DNA from *C. jejuni* (UA580) as DNA probe at 42°C.

UA60, *C. fetus* subsp. *fetus* ATCC 27374; UA487, *C. laridis*; UA580, *C. jejuni* NCTC 11168; and UA578, *C. coli* NCTC 11353. Columns 1 to 7 represent 4 fold serial dilutions of cell suspensions.


1
2
3
4
5
6
42°C
UA60
UA487
UA580
UA578
37°C
UA60
UA487
UA580
UA578

with 2.5 to 5.0 x 10^7 CFU/dot. Columns 2 to 6 were spotted with four-fold serial dilutions of the cell suspensions used in column 1. The results showed that there was no difference between hybridization at the two stringency conditions. Visual examination of the intensity of the dots compared with the *C. jejuni* (UA580) control showed that *C. fetus* (UA60), *C. laridis* (UA478) and *C. coli* (UA578) showed little homology <5% (or at least 2 dilutions difference in intensity of dots) with the *C. jejuni* DNA probe.

When the total genomic DNA from *C. coli* (UA578) was hybridized with *C. fetus* (UA60), *C. laridis* (UA487), and *C. jejuni* (UA580 and UA579) at high stringency conditions, <5 to 25% homology was observed between the different strains (see Figure 7.2). The *C. coli* strain $29a_1$ showed 100% homology with the *C. coli* DNA probe. In contrast, when sonicated DNA (fragment sizes 500 to 1000 base pairs) from *C. coli* (UA578) was used as the DNA probe, the specificity of the probe was greatly reduced (see Figure 7.3). The difference between *C. coli* (UA578) and *C. jejuni* (UA580) was difficult to distinguish visually. The degree of hybridization of *C. laridis* (UA487) and *C. fetus* (UA60) with *C. jejuni* UA578 increased when sonicated DNA was used as a probe (Figure 7.3) instead of unsheared DNA (Figure 7.2).

Figure 7.2 Autoradiograms of dot blot hybridizations with *C. coli* (UA578) using the total genomic DNA as probe at 42°C.

UA60, *C. fetus* subsp. *fetus* ATCC 27374; UA487, *C. laridis*; $29a_1$, *C. coli* isolated from swine; UA580, *C. jejuni* NCTC 11168; UA579, *C. jejuni* NCTC 11392; UA578, *C. coli* NCTC 11353. Columns 1 to 7 represent 4 fold serial dilutions of cell suspensions.

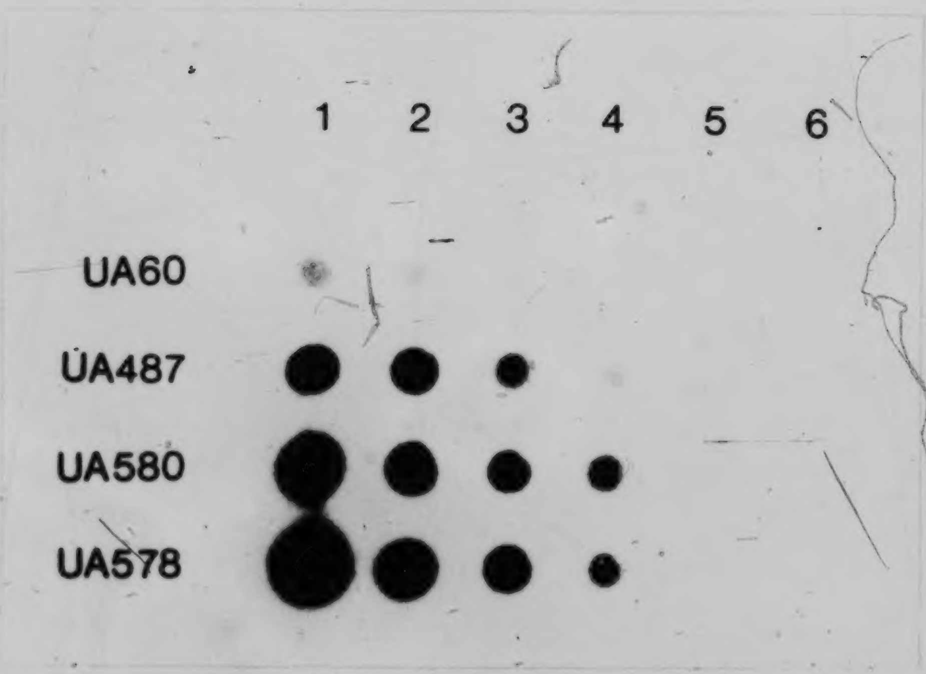
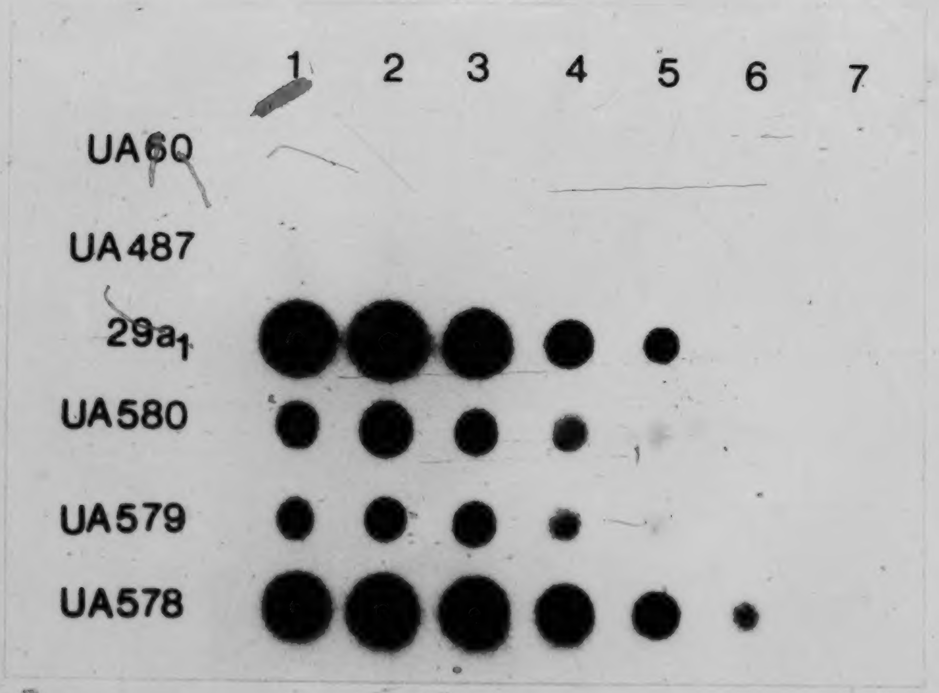

1
2
3
4
5
6
UA60
UA487
UA580
UA578

Figure 7.3 Autoradiograms of dot blot hybridizations with DNA probe prepared from sonicated total genomic DNA from *C. coli* (UA578) at 42°C.

UA60, *C. fetus* subsp. *fetus* ATCC 27374; UA487, *C. laridis*; UA580, *C. jejuni* NCTC 11168; UA578, *C. coli* NCTC 11353. Columns 1 to 7 represent 4 fold serial dilutions of cell suspensions.


1
2
3
4
5
6
7
UA60
UA487
29a1
UA580
UA579
UA578

7.4 Discussion

The dot blot hybridization procedure has an advantage over the slot blot hybridization described and used in Chapter 6, because it does not require the isolation of pure DNA. The isolation of pure DNA from bacteria requires at least one day, therefore, the dot blot procedure is much less time consuming. The method used in this study is sufficiently sensitive to distinguish between *C. coli* and *C. jejuni* provided the DNA was not sheared, for example by sonication. Both *C. coli* and *C. jejuni* can be easily distinguished from other *Campylobacter* species, such as *C. fetus* and *C. laridis*. In comparison with the slot blot technique, both methods differentiate between *Campylobacter* spp., and the degree of homology between the four species tested was similar. Therefore, the dot blot hybridization can be used as a reliable method for differentiating between *Campylobacter* spp. Totten *et al.* (1985) used a dot blot procedure to differentiate *Campylobacter cinaedi* and *C. fennelliae*. In their procedure, only one cell concentration was used, so that more than one hybridization at different stringency conditions was necessary to differentiate closely related strains. In this study, the specificity of the DNA probes was similar when hybridization was carried out at either 37 or 42°C. Therefore, using a serial dilution, the results are easy to interpret and only one stringency condition is required for hybridization. The serial dilutions used can be adjusted for organisms with different degrees of homology. Because the specificity of DNA probe is reduced when sonicated DNA is used as the probe, the isolation of DNA and nick translation procedures should be controlled to obtain consistent results.

In this study, *C. coli* and *C. jejuni* had higher homology with each other than with *C. laridis* and *C. fetus*. This agrees with other DNA homology studies (Hébert *et al.*, 1984; Owen and Leaper, 1981; Roop *et al.*, 1984; Ursing *et al.*, 1983) in which *C. coli* and *C. jejuni* demonstrated 25 to 49% interspecies DNA homology. These two species had about 10% homology with *C. laridis* and about 5% homology with *C. fetus* (Owen and Leaper, 1981; Roop *et al.*, 1984; Ursing *et al.*, 1983).

7.5 Bibliography

Baumann, P., A. L. Furniss, and J. V. Lee. 1985. *Vibrio*, p. 518-538. *In* N. R. Krieg (ed.), Bergey's manual of systematic bacteriology, vol. 1. Williams and Wilkins Co., Baltimore.

Hébert, G. A., P. Edmonds, and D. J. Brenner. 1984. DNA relatedness among strains of *Campylobacter jejuni* and *Campylobacter coli* with divergent serogroup and hippurate reactions. J. Clin. Microbiol. 20:138-140.

Johnson, J. L. 1985. Nucleic acids in bacterial classification, p. 8-11. *In* N. R. Krieg (ed.), Bergey's manual of systematic bacteriology, Vol. 1. Williams and Wilkins Co., Baltimore.

Kafatos, F. C., C. W. Jones, and A. Efstratiadis. 1979. Determination of nucleic acid sequence homologies and relative concentration by a dot hybridization procedure. Nucleic Acids Res. 7:1541-1552.

Maniatis, T., A. Jeffrey, and D. G. Kleid. 1975. Nucleotide sequence of the rightward operator of phage lambda. Proc. Natl. Acad. Sci. U.S.A. 72:1184-1188.

Owen, R. J., and S. Leaper. 1981. Base composition, size and nucleotide sequence similarities of genome deoxyribonucleic acids from species of the genus *Campylobacter*. FEMS Microbiol. Lett. 12:395-400.

Roop, R. M., II, R. M. Smibert, J. L. Johnson and N. R. Krieg. 1984. Differential characteristics of catalase-positive campylobacters correlated with DNA homology groups. Can. J. Microbiol. 30:938-951.

Smibert, R. M. 1985. *Campylobacter*, p.111-118. *In* N. R. Krieg (ed.), Bergey's manual of systematic bacteriology, vol. 1. Williams and Wilkins Co., Baltimore.

Totten, P. A., C. L. Fennell, F. C. Tenover, J. M. Wezenberg, P. L. Perine, W. E. Stamm, and K. K. Holmes. 1985. *Campylobacter cinaedi* (sp. nov.) and *Campylobacter fennelliae* (sp. nov.): Two new *Campylobacter* species associated with enteric disease in homosexual men. J. Infect. Dis. 151:131-139.

Ursing, J., M. Walder, and K. Sandstedt. 1983. Base composition and sequence homology of deoxyribonucleic acid of thermotolerant *Campylobacter* from human and animal sources. Curr. Microbiol. 8:307-310.

8. Comparison of the susceptibilities of *Campylobacter coli* and *Campylobacter jejuni* to antibiotics

8.1 Introduction

The isolation methods developed by Butzler *et al.* (1972) and Skirrow (1977) led to the recognition of *Campylobacter jejuni* and *Campylobacter coli* as important enteric pathogens. Subsequently, other selective media have been developed (Blaser *et al.*, 1979; Bolton *et al.*, 1984; Bolton and Robertson, 1982; Goossens *et al.*, 1983; Karmali and Fleming, 1979; Karmali *et al.* 1986; Lauwers *et al.*, 1978; Rosef *et al.*, 1983; Waterman *et al.*, 1984; Wesley *et al.*, 1983). The media have been developed to overcome the problem of overgrowth by contaminating bacteria, such as members of the family *Enterobacteriaceae*, *Pseudomonas* spp., *Streptococcus* spp., and yeasts (Bolton *et al.* 1983). Most of these media contain blood which is of variable quality and relatively expensive (Bolton *et al.*, 1984). The recent development of blood-free media may be more economical and may lead to media that give more consistent growth of *Campylobacter* strains (Bolton *et al.*, 1984; Karmali *et al.*, 1986). In the blood-free selective media (Hutchinson and Bolton, 1984; Karmali *et al.*, 1986), cefoperazone was used as the selective agent. However, the effect of cefoperazone on the cephalothin-susceptible *Campylobacter* strains (Karmali and Skirrow, 1984; Brooks *et al.*, 1986) has not been determined.

A previous study (Ng *et al.*, 1985; see Chapter 5) on the inhibitory effect of antibiotics to *Campylobacter* species showed that some laboratory strains of *C. coli* were more susceptible to antibiotics used in selective media than strains of *C. jejuni*. This may, in part, account for the lower isolation of *C. coli* compared with *C. jejuni* from stool specimens from patients with gastroenteritis. To test this hypothesis, the effects of antibiotics on fresh isolates of *C. coli* and *C. jejuni* were determined. This was done by determining the Minimal Inhibitory Concentrations (MICs) of a range of antibiotics used in selective media as well as by comparing the counts on non-selective and selective media.

8.2 Materials and methods

8.2.1 Cultures

The following type cultures were used as reference strains for MIC determinations: *C. coli* NCTC 11353, *C. jejuni* NCTC 11168, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* (Oxford strain). All of the *Campylobacter* reference strains were stored as previously described (see Chapter 5). They were subcultured on Mueller-Hinton (MH) agar (Oxoid Ltd., Basingstoke, England) twice before MICs were determined. The *Campylobacter* cultures were incubated for 24 h at 37°C in an atmosphere containing 7% CO_2. Other species were subcultured in a similar manner on nutrient agar and incubated aerobically at 37°C. For comparison of growth on different selective media, laboratory strains of *C. jejuni* UA1, *C. jejuni* UA526, *C. coli* UA421 and *C. coli* UA530 from our previous study (Ng *et al.*, 1985; see Chapter 5) were also included for cross reference of the data for fresh isolates and laboratory strains.

The fresh isolates of *C. coli* and *C. jejuni* used in this study were from animals with either known or unknown antibiotic history (see Chapter 6). Altogether, there were 53 *C. jejuni* biotype I and six *C. jejuni* biotype II isolates from cattle; as well as one *C. jejuni* biotype II, 96 *C. coli* biotype I and five *C. coli* biotype II isolates from swine. After purification by minimal subculturing on Mueller-Hinton (MH) agar (Oxoid Ltd., Basingstoke, England), these isolates were used immediately for MIC determinations or stored at -70°C in sterile preservation medium until use.

8.2.2 Antibiotics

Antibiotics added to selective media and included in the MIC determinations were bacitracin, cephalothin, colistin, polymyxin B sulfate, novobiocin, rifampicin, trimethoprim, vancomycin (all purchased from Sigma, St. Louis, MO, U.S.A.) and cefoperazone (Cefobid®; Pfizer Canada Inc., Kirkland, Quebec). Cycloheximide and amphotericin B used as anti-fungal agents in some selective media were also purchased from Sigma.

8.2.3 MIC determinations

The MICs were determined as described previously (Ng *et al.*, 1985; see Chapter 5).

8.2.4 Effect of selective media

Selected *C. coli* and *C. jejuni* strains were chosen from the 161 cultures used in the MIC determinations to provide a range of organisms with different antibiotic resistance patterns. The strains were grown in MH broth (Oxoid) at 37°C for 24 h in a CO_2 incubator containing 7% CO_2. Serial dilutions of the cultures ranging from 10^{-1} to 10^{-6} were prepared with 0.85% saline. A 0.3 mL aliquot of each dilution, including the undiluted 24-h culture, was transferred to a well of Steers replicator. The Steers replicator with 3 mm diameter pins was used to inoculate approximately 5 µL of each dilution onto the selective media. Inoculated plates were incubated at 42°C in jars containing an atmosphere of 5% CO_2, 10% CO_2 and 85% H_2. Each jar contained an opened petri dish with 3 mL of glycerol on blotting paper to prevent condensation of moisture on the surface of the agar (Stern, 1982). The plates were examined after 20 and 40 h of incubation, and spots containing 15 to 200 colonies were counted under a stereo-microscope. The experiment was conducted in triplicate.

The selective media used in this study are listed in Table 8.1. Each medium was prepared following the procedure described by the authors, unless otherwise stated. The

Table 8.1 The selective media used for quantitative comparisons of the inhibitory effects of antibiotics on the growth of *C. coli* and *C. jejuni*

Media (References)		Antibiotics
M1	(Skirrow, 1977)	vancomycin, polymyxin B, trimethoprim
M2	(Karmali and Fleming, 1979)	vancomycin, polymyxin B, trimethoprim
M3	(Blaser *et al.*, 1979)	vancomycin, polymyxin B, trimethoprim, cephalothin, amphotericin B
M4	(Dekeyser *et al.*, 1972)	polymyxin B, bacitracin, novobiocin, actidione
M5	(Lauwers *et al.*, 1978)	colistin, cephalothin, novobiocin, actidione
M6	(Wesley *et al.*, 1983)	polymyxin B, rifampicin, cefsulodin
M7	(Goossens *et al.*, 1983)	cefoperazone, rifampicin, amphotericin B
M8	(Rosef *et al.*, 1983)	colistin, cephalothin, amphotericin B
M9	(Patton *et al.*, 1981)	colistin, cephalothin, bacitracin, novobiocin, actidione
M10	(Karmali *et al.*, 1986)	cefoperazone, vancomycin, actidione
M11	(Hutchinson and Bolton, 1984)	cefoperazone
M12	(Waterman *et al.*, 1984)	polymyxin B, trimethoprim, rifampicin, actidione

medium developed by Lauwers *et al.* (1978) was prepared without blood or with 10% sheep blood. MH agar was used as the reference medium.

8.3 Results

8.3.1 MIC determinations

The MICs of antibiotics for 101 *C. coli* and 60 *C. jejuni* strains are summarized in Table 8.2. The MICs of bacitracin, trimethoprim and vancomycin were similar for all of the strains tested. A greater range in susceptibility to other antibiotics was noted for both *C. jejuni* and *C. coli*. All of the strains tested were resistant to the levels of antibiotics used in selective media. The strains with MICs of cephalothin from 32 to 64 µg/mL were further tested for susceptibility to cefoperazone. All of these strains had MICs of cefoperazone >128 µg/mL. Strains with MICs of cephalothin of 8 µg/mL had MICs of cefoperazone of 64 to 128 µg/mL. Therefore, moderate susceptibility to cepholothin in certain strains did not correlate with the susceptibility to cefoperazone. In contrast, cephalothin-susceptible *C. coli* UA421 from our previous study (Ng *et al.*, 1985) and a cephalothin-susceptible derivative of *C. jejuni* strain from this study both had MICs of 4 µg/mL to cephalothin and 32 µg/mL to cefoperazone. Therefore, these 2 strains were susceptible to the level of cefoperazone added to some selective media (Goossens *et al.*, 1983; Bolton *et al.*, 1984;

Table 8.2 Range of MICs for 101 strains of *C. coli* and 60 strains of *C. jejuni* to eight antibiotics used in selective media for isolation of *Campylobacter* spp.

	MIC (µg/mL)[a]					
	C. coli			*C. jejuni*		
Antibiotics	Range	50%	90%	Range	50%	90%
Bacitracin[b]	256->512	>512	>512	256->512	512	>512
Cephalothin	32->256	256	256	64-128	128	128
Colistin[c]	<1-16	2	4	1-16	4	8
Novobiocin	8-128	16	64	16-128	32	32
Polymyxin B[d]	<1-8	<1	2	<1-8	<1	4
Rifampicin	16->128	128	128	4-128	16	32
Trimethoprim	256->256	>256	>256	256->256	256	>256
Vancomycin	128->128	>128	>128	128->128	>128	>128

[a] 50% and 90%, MICs for 50 and 90% of the strains, respectively

[b] 1 µg = 65.2 IU

[c] 1 µg = 13.6 IU

[d] 1 µg = 8 IU

Karmali *et al*., 1986).

Based on the MIC data, six pairs of strains of *C. coli* (six isolates from the filtration method and six isolates from selective media isolation method) and three pairs of *C. jejuni* strains were selected for further study. The MICs of polymyxin, colistin, rifampin, cephalothin and novobiocin of these 12 strains of *C. coli* and 6 strains of *C. jejuni* are shown in Tables 8.3 and 8.4. MICs of laboratory strains of two *C. coli* and two *C. jejuni* used in a previous study (Ng *et al*., 1985, see Chapter 5) were also included for cross reference of the data for fresh isolates and laboratory strains.

8.3.2 Effect of antibiotic combinations

The resistance of the selected *Campylobacter* strains to the combinations of antibiotics used in selective media was compared on MH agar. The number of colonies (CFU/mL) growing on MH agar was compared with the number of colonies of growth on MH with antibiotic combinations. The differences in the $\log_{10}$ CFU/mL on MH agar and each selective medium are shown in Table 8.5. All of the *C. jejuni* strains grew well on all of the selective media, except on medium M5 without blood and medium M9. The greatest level of inhibition was a 5 log cycle decrease in count for *C. jejuni* strains UA1 and UA526. The addition of blood to medium M5 eliminated the inhibitory effect. Medium M9 only inhibited *C. jejuni* UA526.

Table 8.3 The range of MICs of 14 *C. coli* strains to five antibiotics used in selective media for the isolation of *Campylobacter* spp.

Strain[a]	Antibiotic[b] PB	 CL	 RA	 CF	 NB
			MIC (µg/mL)		
C. coli					
UA421	2-4	16	2	8	≤1
UA530	8	8-16	32-64	128-256	64
C. coli biotype I					
$51a_2$	≤1	4	64	256	32
$51b_1$	≤1	4	128	256	32
$65a_1$	≤1	≤1	64	256	32
$65b_1$	≤1	2	128	256	16
$74a_2$	4	8	128	256	64
74b	14	8	128	256	64
76a	≤1	1	128	16-32	64
76b	≤1	1	128	32	64
$81a_2$	2	2	>128	>256	128
$81b_2$	≤1	1	>128	128	128
C. coli biotype II					
48a	≤1	4	64	256	32
48b	≤1	4	64	256	32

[a] Isolates from MH agar using the filtration technique (Dekeyser *et al.*, 1972) are indicated by "a" in the strain number, those isolates "b" are isolated from MH agar with trimethoprim, polymyxin B and vancomycin (Skirrow, 1977).

[b] PB, polymyxin B sulfate; CL, colistin; RA, rifampicin; CF, cephalothin; NB, novobiocin.

Table 8.4 The range of MICs of 8 *C. jejuni* strains to five antibiotics used in selective media for the isolation of *Campylobacter* spp.

Strain[a]	PB	CL	RA	CF	NB
			Antibiotic[b]		
			MIC (µg/mL)		
C. jejuni					
UA1	8	16-32	32	64-128	128
UA526	8	16	128	128-256	32
C. jejuni biotype I					
$15a_1$	8	16	128	128	32
$15b_1$	4	8	128	128	32
$16a_2$	≤1	4	4	64	32
$16b_1$	4	4	16	64	16
C. jejuni biotype II					
$9a_2$	≤1	8	4	64-128	32
$9b_1$	4	4	8	64-128	32

[a] Isolates from MH agar using the filtration technique (Dekeyser *et al.*, 1972) are indicated by "a" in the strain number, those isolates "b" are isolated from MH agar with trimethoprim, polymyxin B and vancomycin (Skirrow, 1977).

[b] PB, polymyxin B sulfate; CL, colistin; RA, rifampicin; CF, cephalothin; NB, novobiocin.

Table 8.5 Inhibitory effect of selective media on *C. coli* and *C. jejuni*

	Differences in $\log_{10}$ CFU/mL[a]												
Strains	M1	M2	M3	M4	M5	M5B[b]	M6	M7	M8	M9	M10	M11	M12
C. coli													
48a	<1	1	1	4	1	<1	≥5	2	<1	4	1	<1	<1
48b	<1	1	1	5	1	<1	≥5	2	<1	4	3	<1	<1
$51a_2$	<1	<1	<1	4	1	<1	5	1	<1	4	1	<1	<1
$51b_1$	<1	<1	<1	4	1	<1	5	1	<1	4	<1	<1	<1
$65a_1$	<1	1	<1	≥5	1	<1	5	4	<1	≥5	5	<1	<1
$65b_1$	<1	2	2	≥5	2	<1	5	1	<1	≥5	3	<1	<1
$74a_2$	<1	<1	<1	3	1	<1	1	<1	<1	3	1	<1	<1
74b	<1	<1	<1	3	1	1	1	<1	<1	3	1	<1	<1
76a	<1	<1	<1	4	2	<1	4	<1	<1	<1	1	<1	<1
76b	<1	<1	<1	4	2	<1	5	<1	<1	<1	<1	<1	<1
$81a_2$	1	3	<1	6	4	4	6	4	3	6	5	<1	1
$81b_2$	<1	2	1	5	4	2	6	3	2	5	3	<1	1
UA421	<1	<1	5	≥5	≥5	≥5	≥5	≥5	≥5	≥5	4	≥5	<1
UA530	<1	<1	<1	<1	5	<1	<1	<1	<1	<1	3	2	<1
C. jejuni													
$9a_2$	<1	<1	<1	<1	1	<1	1	<1	<1	ND[c]	<1	1	<1
$9b_1$	<1	<1	<1	<1	<1	<1	<1	<1	<1	ND	1	1	<1
$15a_1$	1	<1	<1	1	<1	<1	<1	1	<1	ND	<1	<1	<1
$15b_1$	1	<1	1	<1	1	<1	1	1	<1	ND	<1	1	<1
$16a_2$	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
$16b_1$	<1	<1	<1	<1	1	<1	<1	<1	<1	<1	<1	<1	<1
UA1	<1	<1	<1	<1	5	<1	<1	<1	<1	<1	<1	<1	<1
UA526	<1	<1	<1	2	5	<1	1	<1	<1	4	1	<1	<1

[a] Log_{10} count on MH agar minus $\log_{10}$ count on selective medium, corrected to the nearest $\log_{10}$ difference

[b] M5B refer to medium M5 with blood

[c] ND: not done

The *C. coli* strains grew well on medium M1 and M12. Only the cephalothin-susceptible strain UA421 was inhibited by medium M11. This strain was inhibited by most media. Medium M6 was the most inhibitory to the *C. coli* isolates, counts for ten out of the twelve strains were reduced by at least 5 log cycles. Growth of all of the *C. coli* strains was inhibited on at least one of the culture media. Medium M5 inhibited all of the *C. coli* strains tested. With the addition of blood, the inhibitory effect against some strains was reduced. Only *C. coli* strains 76a and 76b were not inhibited by medium M9, the counts of all other *C. coli* strains were reduced by at least 3 log cycles on this medium. When the inhibitory effects of the selective media on the isolates obtained by the filtration method and the selective media method were compared, no significant difference was observed as a result of the method of isolation (Table 8.5). For example, the growth response of *C. coli* strains 48a and 48b were similar on all of the media, except medium M10.

8.4 Discussion

When fresh isolates of *C. coli* and *C. jejuni* were examined, all the isolates tested showed a high level of resistance to trimethoprim, vancomycin and bacitracin. The MICs for the other antibiotics tested showed a wider range among the *C. coli* and *C. jejuni* strains, especially for rifampicin. The MIC results with fresh isolates of *C. coli* parallelled those found for laboratory strains of *C. coli* (Ng *et al.*, 1985; Chapter 5). Some of the fresh isolates of *C. jejuni* were more susceptible to cephalothin (MIC 64 µg/mL) and rifampicin (MIC 4 µg/mL) than the laboratory strains (Ng *et al.*, 1985; Chapter 5).

For the evaluation of the inhibitory effects of selective media, laboratory strains of *C. coli* (UA421 and UA530) and *C. jejuni* (UA1 and UA526) from the previous study (Ng *et al.*, 1985; Chapter 5) were included. The results obtained in this study were comparable to the results obtained in the previous study. For example, the cephalothin-susceptible *C. coli* strain UA421 did not grow on most of the selective media tested in either study, whereas *C. jejuni* UA1 grew well on most of the media tested. This indicated that the method used in this study for the evaluation of the inhibitory effect of selective media for *Campylobacter* strains is comparable to the plate count method used previously (Chapter 5). This method is less laborious and more economical than the conventional plate count method.

Medium M1 which contained polymyxin B sulfate, trimethoprim and vancomycin (Skirrow, 1977) and the medium M12 which contained cefoperazone, vancomycin and actidione (Karmali *et al.*, 1986) were the least inhibitory of the selective media for both the *C. coli* and *C. jejuni* strains tested. Medium M11 which only contained cefoperazone (Hutchinson and Bolton, 1984) inhibited the growth of the cephalothin-susceptible *C. coli* UA421, although this strain grew well on M12. The MIC of cefoperazone for UA421 was 32 µg/mL. Therefore, besides the antibiotics added to M11 and M12, other constituents in the media may affect the growth of *C. coli* UA421. Both M11 and M12 contain charcoal and sodium pyruvate, but only M12 contains hemin (Hutchinson and Bolton; Karmali *et al.*, 1986). The basal media used in M11 and M12 are different (Hutchinson and Bolton; Karmali *et al.*, 1986). Therefore, hemin and other components in media M12 may have a protective effect on the cephalothin-susceptible strain. Substituting cephalothin with cefoperazone may reduce the contaminants (Hutchinson and Bolton, 1984; Karmali *et al.*, 1986) but it may still inhibit the growth of *Campylobacter* strains which were susceptible to cephalothin because these strains have MIC of ≤32 µg/mL for cefoperazone.

Similar to our previous study (Ng *et al.*, 1986), the most inhibitory medium in this study was medium M6 which contained polymyxin B (20,000 IU/L), rifampicin (25 mg/L) and cefsulodin (6.25 mg/L) (Wesley *et al.*, 1983). This may be due to the high concentration of polymyxin B sulfate in the medium (Lovett *et al.*, 1983). Medium M5 with blood (Lauwers *et al.*, 1978) and medium M9 (Patton *et al.*, 1981) contain the same antibiotics, but M9 contains a higher concentration of colistin (40,000 IU/L) than M5 (10,000 IU/L). With this increased level of colistin, ten out of the twelve strains of *C. coli* were inhibited, and one of the four strains of *C. jejuni* was also inhibited. This might be expected, as polymyxin B and colistin have similar modes of action and inhibitory effects on *Campylobacter* strains.

The effects of selective media on cultures isolated either using the filtration method (Dekeyser *et al.*, 1972) or from antibiotic plates (Skirrow, 1977) were similar. This indicated that use of the selective medium (MH with Skirrow's antibiotics) for isolation did not select for strains with increased resistance to antibiotics. Based on the results of this study and the previous study (Ng *et al.*, 1985, Chapter 5), fresh isolates of *C. coli* were similar to laboratory strains in susceptibility to selective media used for isolating *Campylobacter* spp. Some of the selective media would be too inhibitory for enumerating or isolating *C. coli* from the extra-enteral environment, such as water and foods.

Bibliography

Beuchat, L. R. 1985. Efficacy of media and methods for detecting and enumerating *Campylobacter jejuni* in refrigerated chicken meat. Appl. Environ. Microbiol. 50:934-939.

Blaser, M. J., I. D. Berkowitz, F. M. LaForce, J. Cravens, L. B. Reller, and W.-L. L. Wang. 1979. Campylobacter enteritis: Clinical and epidemiologic features. J. Intern. Med. 91:179-185.

Bolton, F. J., D. Coates, P. M. Hinchliffe, and L. Robertson. 1983. Comparison of selective media for isolation of *C. jejuni/coli*. J. Clin. Pathol. 36:78-83.

Bolton, F. J., D. N. Hutchinson, and D. Coates. 1984. Blood-free selective medium for isolation of *C. jejuni* from feces. J. Clin. Microbiol. 19:169-171.

Bolton, F. J., and L. Robertson. 1982. A selective medium for isolating *Campylobacter jejuni/coli*. J. Clin. Pathol. 35:462-467.

Bopp, C. A., J. G. Wells, and T. J. Barrett. 1982. Trimethoprim activity in media selective for *Campylobacter jejuni*. J. Clin. Microbiol. 16:808-812.

Dekeyser P., M. Gossuin-Detrain, J. P. Butzler, and J. Sternon. 1972. Acute enteritis due to related vibrio: First positive stool cultures. J. Infect. Dis. 125:390-392.

Hutchinson, D. N., and F. J. Bolton. 1984. Improved blood free selective medium for the isolation of *Campylobacter jejuni* from faecal specimens. J. Clin. Pathol. 37:956-957.

Karmali, M. A., and P. C. Fleming. 1979. Campylobacter enteritis in children. J. Pediatr. 94:527-533.

Karmali, M. A., A. E. Simon, M. Roscoe, P. C. Fleming, S. S. Smith, and J. Lane. 1986. Evaluation of a blood-free, charcoal-based, selective medium for the isolation of *Campylobacter* organisms from feces. J. Clin. Microbiol. 23:456-459.

Karmali, M. A., and M. B. Skirrow. 1984. Taxonomy of the genus *Campylobacter*, p. 1-20. *In* J. P. Butzler (ed.), Campylobacter infection in man and animals. CRC Press, Inc., Boca Raton, Fla.

Lauwers, S., M. De Boeck, and J. P. Butzler. 1978. Campylobacter enteritis in Brussels. Lancet i:604-605.

Merino, F. J., A. Agulla, P. A. Villasante, A. Diaz, J. V. Saz, and A. C. Velasco. 1986. Comparative efficacy of seven selective media for isolating *Campylobacter jejuni*. J. Clin. Microbiol. 24:451-452.

Patton, C. M., S. W. Mitchell, M. E. Potter, and A. F. Kaufmann. 1981. Comparison of selective media for primary isolation of *Campylobacter fetus* subsp. *jejuni*. J. Clin. Microbiol. 13:326-330.

Ng, L.-K., M. E. Stiles, and D. E. Taylor. 1985. Inhibition of *Campylobacter coli* and *Campylobacter jejuni* by antibiotics used in selective media. J. Clin. Microbiol. 22:510-514.

Rosef, O., B. Gondrosen, G. Kapperud, and B. Underdal. 1983. Isolation and characterization of *Campylobacter jejuni* and *Campylobacter coli* from domestic and wild mammals in Norway. Appl. Environ. Microbiol. 46:855-859.

Skirrow, M. B. 1977. Campylobacter enteritis: A "new" disease. Br. Med. J. 2:9-11.

Steers, E., E. L. Foltz, and B. S. Graves. 1959. Inocula replicating apparatus for routine testing of bacterial susceptibility to antibiotics. Antibiot. Chemother. 9:307-311.

Stern, N. J. 1982. Selectivity and sensitivity of three media for recovery of inoculated *Campylobacter fetus* ssp. *jejuni* from ground beef. J. Food Safety 4:169-175.

Waterman, S. C., R. W. A. Park, and A. J. Bramley. 1984. A search for the source of *Campylobacter jejuni* in milk. J. Hyg. (*Camb.*) 92:333-337.

Wesley, R. D., B. Swaminathan, and W. J. Stadelman. 1983. Isolation and enumeration of *Campylobacter jejuni* from poultry products by a selective enrichment method. Appl. Environ. Microbiol. 46:1097-1102.

9. Use of DNA probes to study tetracycline-resistance in *Campylobacter* from swine and cattle

9.1 Introduction

Epidemiological studies of *Campylobacter jejuni* and *Campylobacter coli* have used plasmid profiles as a parameter for strain differentiation (Austen and Trust, 1980; Bradbury *et al.*, 1983; Bradbury and Munroe, 1985; Taylor *et al.*, 1983b; Tenover *et al.*, 1983, 1985). The function of most of these plasmids is unknown. In contrast, plasmids which encode tetracycline-resistance (Tc^R) have been extensively studied. Taylor *et al.* (1983a) reported that Tc^R was encoded on a 38 Mdal plasmid in both *C. coli* and *C. jejuni*. More recently, these plasmids have been shown to be about 45 kilobases (kb) (Taylor *et al.*, 1986). Lambert *et al.* (1985) reported a 47.2 kb plasmid, pIP1433, in *C. coli* which encodes both Tc^R and kanamycin-resistance.

Conjugative plasmids encoding Tc^R in six isolates of *Campylobacter* from Canada, Belgium and United States have a high degree of homology (Taylor *et al.*, 1983a). However, these same plasmids did not share DNA homology with the four classes of Tc^R determinants (A, B, C and D) found in *Enterobacteriaceae* (Mendez *et al.*, 1980; Taylor *et al.*, 1983a). In a *C. jejuni* plasmid pUA466, the region responsible for Tc^R included a 4.2 kb *Acc*I fragment and a 1.8 kb *Hinc*II fragment (Figure 9.1), and these fragments are conserved in all of the *Campylobacter* plasmids studied so far (Taylor *et al.*, 1986). The plasmid pUA649 in which the 4.2 kb *Acc*I fragment was deleted from pUA466 resulted in a tetracycline-susceptible (Tc^S) strain (Taylor, 1986). The Tc^R determinant from pUA466 was cloned and expressed in *Escherichia coli* (Taylor, 1986). Moreover, the *Campylobacter* Tc^R determinant was shown to have homology with a 5 kb fragment containing the *tetM* determinant in the plasmid pJI3 from *Streptococcus agalactiae* B109 (Taylor, 1986) (see Figure 9.1). This *tetM* determinant has also been found in a number of unrelated organisms, such as *Ureaplasma urealyticum*, *Mycoplasma spp.* and *Gardnerella vaginalis* (Roberts and Kenny, 1986).

Classification of Tc^R determinants in gram-positive and gram-negative bacteria was based on DNA hybridization studies using Tc^R DNA probes (Burdett *et al.*, 1982; Levy, 1984; Mendez *et al.*, 1980). In this study, probes from the Tc^R determinant in pUA466, namely a 1.8 kb *Hinc*II fragment, as well as the 5 kb fragment which encodes Tc^R from the plasmid pJI3 in *S. agalactiae* (Roberts and Kenny, 1986) were used to study the heterogeneity of Tc^R isolates from swine and cattle.

9.2 Materials and methods

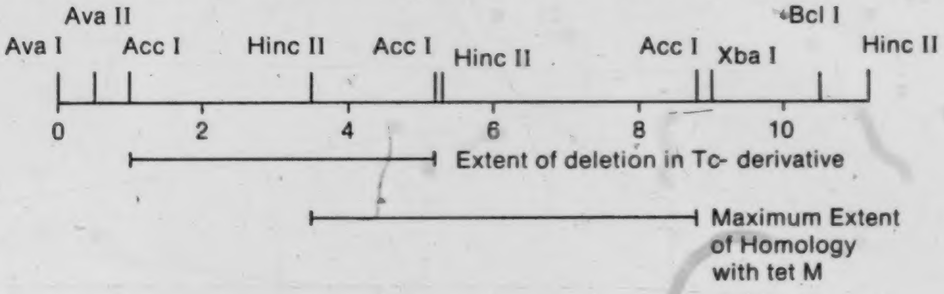
9.2.1 Bacterial strains

The Tc^R strains of *C. jejuni* and *C. coli* isolated from colons of cattle and swine (described in Chapter 6) were selected, including 9 strains of *C. jejuni* and 18 strains of

Figure 9.1 The restriction map of *C. jejuni* Tc^R region of the plasmid pUA466.

The deletion Tc-derivative of pUA466 is named pUA649.

Restriction Map of Tetracycline Resistance Region of pUA 466


Ava II
Bcl I
Ava I
Acc I
Hinc II
Acc I
Hinc II
Acc I
Xba I
Hinc II
0
2
4
6
8
10
Extent of deletion in Tc- derivative
Maximum Extent of Homology with tet M

C. coli. All of the nine *C. jejuni* strains and three of the *C. coli* Tc^R strains were from four animals which had not been exposed to antibiotics, either in their feed or for therapeutic purposes. In addition, 15 Tc^S *C. coli* and *C. jejuni* strains isolated from cattle and swine were selected for comparison with the Tc^R strains. *Campylobacter* strains used as controls or as recipients for mating experiments are listed in Table 9.1.

9.2.2 Restriction endonucleases

*Acc*I, *Xba*I, *Xho*I, *Hin*dIII, *Hin*cII and *Kpn*I were obtained from Boehringer Mannheim Canada Ltd. (Dorval, Quebec). Enzyme digestions were performed according to the manufacturer's instructions.

9.2.3 Plasmid DNA isolation

A modified Birnboim and Doly procedure (1979) was used to prepare purified plasmid DNA. An overnight culture of *Campylobacter* test organsism was grown on 15 Mueller-Hinton (MH) agar plates and harvested by scraping the cells off the plates into 50 mL of TE buffer (50 mM Tris and 10 mM EDTA, pH 8.0). The pellet of cells obtained by centrifugation at 10,000 rpm for 10 min (Rotor JA20, Beckman Instruments, Inc., Fullerton, CA, U.S.A.) was resuspended in 1.7 mL of solution I (50 mM glucose, 10 mM EDTA, 0.25 M Tris, pH 8.0 and 2 mg/mL lysozyme) and held on ice for 30 minutes. Solution I was prepared fresh from stock solutions. The

Table 9.1 Strains of *Campylobacter* used in mating and hybridization experiments.

Organism	Strain no.	Antibiotic resistance	Source
Mating experiment:			
Donor			
C. jejuni	UA466	Tc	human[a]
C. coli	$LK25b_1$	Tc	swine (this study)
Recipient			
C. jejuni	SD2	Nal	human[b]
C. fetus	ATCC 27374	Nal	ATCC
C. laridis	UA487	Nal	human[c]
Hybridization experiment:			
C. jejuni	UA466	Tc	human[a]
C. jejuni	UA649	None	deletion mutant of UA466
C. jejuni	UA650	None	plasmidless derivative of UA466
C. jejuni	UA1	Tc	human[b]
C. jejuni	UA124	none	plasmidless derivative of UA1
C. jejuni	34b	Tc	human[d]
C. coli	44b	Tc	human[d]

[a] Lee, E., Naval Research Institute, Bethesda, MD, U.S.A.

[b] Karmali, M. A., The Hospital for Sick Children, Toronto, Ontario.

[c] Lior, H., Laboratory Centre for Disease Control, Ottawa, Ontario.

[d] Mueller, L., Provincial Laboratory of Public Health, Edmonton, Alberta.

cells were lysed by adding 3.3 mL of solution II (0.2 N NaOH containing 1% sodium dodecyl sulfate). After 5 min on ice, 2.5 mL of solution III (3M sodium acetate, pH 4.8) was added to precipitate the denatured chromosome and proteins. The lysate was held on ice for one hour, and then centrifuged for 15 min at 12,500 rpm (Rotor JA20, Beckman). DNA in the supernatant was precipitated by adding two volumes of ice cold 95% ethanol and held at -20°C for 18 h. The DNA was recovered by centrifugation at 10,000 rpm (Rotor JA20, Beckman) for 10 min. The resulting DNA pellet was dissolved in TE buffer and centrifuged on a Cesium chloride gradient for 22 to 24 h at 55,000 rpm in a Beckman L8 ultracentrifuge (Rotor 70.1Ti).

9.2.4 Plasmid analysis

Organisms were screened for plasmid DNA using the method described in Chapter 6. Fragments of plasmids from restriction endonuclease digestions were subjected to electrophoresis for 18 h in horizontal 0.6% agarose gels in Tris borate-EDTA buffer with digests of phage λ and plasmid R27 (Taylor and Brose, 1985) as molecular weight standards. Plasmid pUA466 in *C. jejuni* UA466 and plasmid pMAK175 in *C. jejuni* UA1 were included as postive controls.

9.2.5 Preparation of ^{32}P-labelled DNA

The 1.8 kb *Hin*cII fragment from pUA466 was cloned into pUC8 to give plasmid pUOA1 in *E. coli* JM105 (Taylor, 1986).

Plasmid pJI3 (pACYC177 with the 5 kb fragment containing the *tetM* determinant from *S. agalactiae*) was supplied by V. Burdett (Duke University Medical Center, NC, U.S.A.). The 1.8 kb *Hin*cII fragment from pUA466 and the 5 kb fragment from pJI3 were prepared by electroelution from agarose gel onto DEAE paper (Dretzen *et al.*, 1981). The DNA fragments and plasmid pUA649 were labelled *in vitro* with [α-^{32}P]dCTP (New England Nuclear Corp. Boston, MA, U.S.A.) by nick translation (Maniatis *et al.*, 1975). The labelled DNA had specific activities of 10^7 to 10^8 cpm/μg.

Dot blot procedure. The dot blot procedure used was described in Chapter 7. The sensitivity and specificity of the 1.8 kb *Hin*cII fragment from pUA466 and the 5 kb tetM probe were determined by using two temperatures of hybridization for each probe to represent two stringency conditions. The stringency of hybridization was determined using the formula by Schildkraut and Lifson (1965) and McConaughy *et al.* (1969):

$$Tm = 81.5 + 16.6\log M + 0.41(G+C) - 0.72(\%\ formamide)$$

where Tm is the melting temperature of DNA, and M is the concentration of Na^+ in solution.

The rate of reassociation of denatured DNA is at maximum at 20 to 25°C below the Tm (Marmur and Doty, 1961). Therefore, hybridization at 37°C was used to represent standard stringency (Tm - 20°C) of hybridization and 42°C was used to represent higher stringency (Tm - 15°C) of hybridization.

Overnight broth cultures in MH broth were concentrated ten-fold to give about 10^9 to 10^{10} CFU/mL. The actual number of cells in these concentrated cultures was determined by a plate count method, so that the number of cells loaded on each dot was known. A range of sample sizes was loaded onto nitrocellulose filters to give 10^5 to 10^7 CFU per dot. The membrane filters were then processed and hybridized with DNA probes.

9.2.6 Southern transfer hybridization

DNA was transferred from agarose gels to nitrocellulose filter membranes (BA85; Schleicher and Schuell Inc., Keene, NH, U.S.A.) by the method of Southern (1975). Hybridizations were performed, as described by Portnoy *et al.* (1981) at 42°C for 16 h. For each blot, 2 to 5 x 10^5 cpm ^{32}P-labelled DNA probe was used.

9.2.7 Minimal Inhibition Concentration (MIC) determination

The MICs of tetracycline for the *Campylobacter* isolates were carried out by the agar dilution method described previously (Ng *et al.*, 1985, see Chapter 5).

9.2.8 Mating experiments

A plate mating method was used for transfer of Tc^R plasmids, as described by Taylor (1981). Antibiotic plates containing nalidixic acid (48 μg/mL) and tetracycline (8 μg/mL) were used to select for transconjugants. Control plates were monitored for spontaneous mutation of either the donor or the recipient strains.

9.2.9 Plasmid curing experiment

Cultures were grown in MH broth at 37°C in an atmosphere of 7% CO_2. A 0.5 mL aliquot of an overnight culture was inoculated into a series of MH broth tubes containing 0.5 to 7 μg of ethidium bromide per mL and incubated at 37°C for 48 h. The tube with the highest concentration of ethidium bromide which showed growth of the cells was plated onto MH agar plates. After 48 h of incubation, the colonies were replica-plated onto fresh MH plates containing tetracycline (8 μg/mL) using sterile velvet. Tc^S colonies were selected for plasmid analysis. When no Tc^S colonies were detected after treating with ethidium bromide, 48 colonies were randomly selected and screened for the absence of plasmids.

9.3 Results

9.3.1 MIC determination

The MICs of tetracycline for the 23 isolates ranged from 16 to >64 μg/mL (see Table 9.2). The MICs for isolates 48a, 48b, and $59b_1$ were 16 μg/mL and for the other isolates, MICs were ≥32 μg/ml.

Table 9.2 The MIC and plasmid content of tetracycline-resistant isolates from the colon of cattle and swine.

Isolates[a]	Source	MIC (µg/mL)	Number of plasmid bands
C. jejuni			
7a	cattle	32	1
$9a_1$ to $9a_7$, 9b	cattle	64	1
C. coli			
1a, 1b	cattle	64	7
$25b_1$	swine	64	2
$29a_1$, a_2, a_3, b	swine	>64	1
48a, 48b	swine	16	1
$59b_1$	swine	16	1
$75a_1$, $75a_2$	swine	64	8
$82a_1$, $82a_2$	swine	>64	1

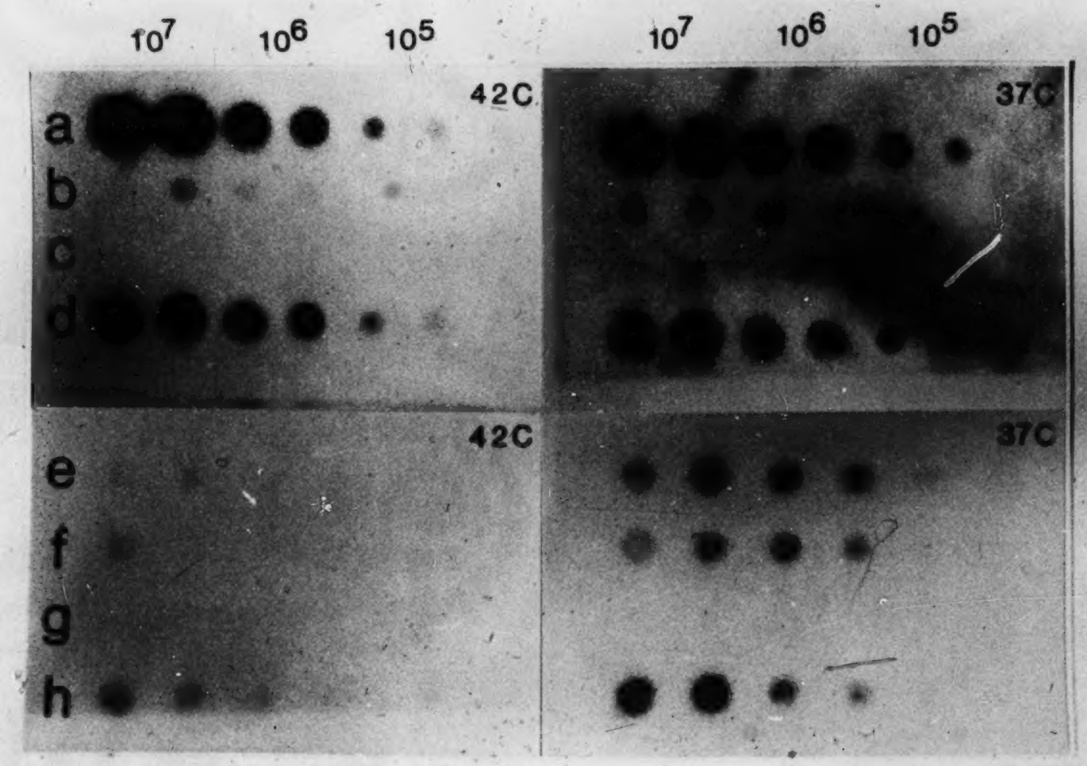
[a] Cultures from the same colon sample have the same number. "a" and "b" indicate that the isolate was obtained from MH or MH with Skirrow's antibiotics, respectively. The subscripts indicate the isolate number from the same agar plate.

9.3.2 Conditions of dot blot hybridization

The sensitivity of the 1.8 kb *Hin*cII fragment and the 5 kb *tetM* probes for the detection of Tc^R was compared using 4 *Campylobacter* strains (UA466, UA649, UA650 and 48a). The results are illustrated in Figure 9.2. The dots in rows a to d were hybridized with the 1.8 kb fragment at 42°C (Figure 9.2, left panel) or 37°C (Figure 9.2, right panel). Row e to h were similarly hybridized with the *tetM* probe. Little difference was observed between hybridization at 42°C and 37°C with this probe. Using the 1.8 kb probe, the minimum number of cells required for the hybridization to be detected by autoradiography was 10^5 cells per dot. When the *tetM* probe was used for hybridization at 42°C, little hybridization was detected, however at 37°C, hybridization was detected. Plasmid pUA649, a Tc^S deletion derivative of pUA466 also hybridized. Although the pUA649 plasmid is missing the majority of the Tc^R determinant (see Figure 9.1), it still retains approximately 400 base pairs from the tetracycline resistance determinant. The lowest detectable level was 10^6 CFU per dot. Therefore, the sensitivity and specificity of the 1.8 kb *Hin*cII fragment is higher than that of the *tetM* probe for detecting Tc^R determinant in *Campylobacter*. For subsequent dot blot experiments, using 1.8 kb *Hin*cII fragment as the probe, hybridization was conducted at 42°C with about 10^5 to 10^6 cells per dot.

Figure 9.2 Comparison of the sensitivity and specificity of two DNA probes: the 1.8 kb *Hinc*II fragment from pUA466 and the 5 kb *tetM* probe from pJI3.

In rows a to ~~d, the probe~~ was the 1.8 kb *Hinc*II fragment and in rows e to h, the probe was the 5 kb *tetM*. The temperatures of hybridization were 42°C (left panels) and 37°C (right panels). The concentration of cells loaded per dot (10^5 to 10^7) are indicated above the panels. UA466, rows a and e; UA649, rows b an f; UA650, rows c and g; 48a, rows d and h.


10⁷
10⁶
10⁵
10⁷
10⁶
10⁵
42C
37C
a
b
c
d
42C
37C
e
f
g
h

9.3.3 Dot blot hybridization with 1.8 kb *Hinc*II fragment and *tetM* probes

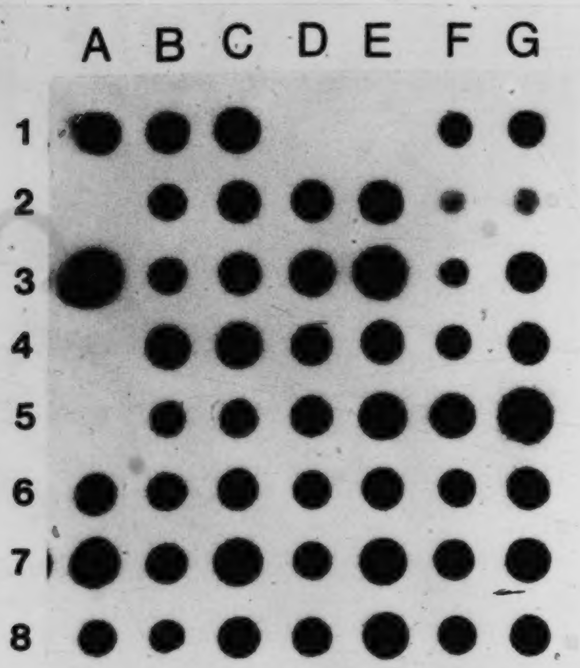
The 23 Tc^R isolates listed in Table 9.2 were hybridized separately with the 1.8 kb *Hinc*II fragment and the 5 kb *tetM* probe. The autoradiograms obtained by hybridization of these isolates with the 1.8 kb probe are shown in Figure 9.3. The Tc^S controls UA124, UA649, UA650 and *C. coli* 2b did not hybridize with the 1.8 kb *Hinc*II fragment. There was no difference between the Tc^R clinical isolates *C. coli* 34b and 44b (obtained from L. Mueller, Alberta Provincial Laboratory of Public Health) and the Tc^R isolates from animals, except for *C. coli* strain $25b_1$, which gave a weak hybridization. When the hybridizations were repeated with 8 x 10^7 to 2 x 10^8 cells of *C. coli* strain $25b_1$ per dot, a strong positive reaction was observed. In contrast, Tc^S isolates of *C. coli* from the same colon, for example *C. coli* $25a_1$, did not hybridize with the 1.8 kb *Hinc*II fragment, using the dot blot procedure. However, *C. coli* strain $25a_1$ also contained a plasmid. The 5 kb *tetM* probe hybridized with all of the Tc^R strains, however, because of the lower sensitivity of the probe, a longer exposure time of X-ray film was required.

9.3.4 Plasmid analysis and Southern transfer hybridization

Most of the Tc^R strains contained a single plasmid of approximately the same mobility in agarose gel as the 45 kb plasmids pUA466 from *C. jejuni* UA466 and pMAK175 from *C.*

Figure 9.3 Dot blot hybridization with 1.8 kb *Hinc*II fragment from pUA466.

Upper panel shows the autoradiogram; lower panel indicates the identity of the cultures. The Tc^S strains are indicated as T^S in the grid.



	A	B	C	D	E	F	G
1	UA1	48b	48b	2b T^s	2b T^s	9b	9b
2	UA124 T^s	59b	59b	7a	7a	$25b_1$	$25b_1$
3	UA466	$74a_1$	$74a_1$	$9a_1$	$9a_1$	$29a_1$	$29a_1$
4	UA649 T^s	$75a_1$	$75a_1$	$9a_2$	$9a_2$	$29a_2$	$29a_2$
5	UA650 T^s	$75a_2$	$75a_2$	$9a_3$	$9a_3$	$29a_3$	$29a_3$
6	34b	79a	79a	$9a_4$	$9a_4$	29b	29b
7	44b	1a	1a	$9a_5$	$9a_5$	$82a_1$	$82a_1$
8	48a	1b	1b	$9a_6$	$9a_6$	$82a_2$	$82a_2$

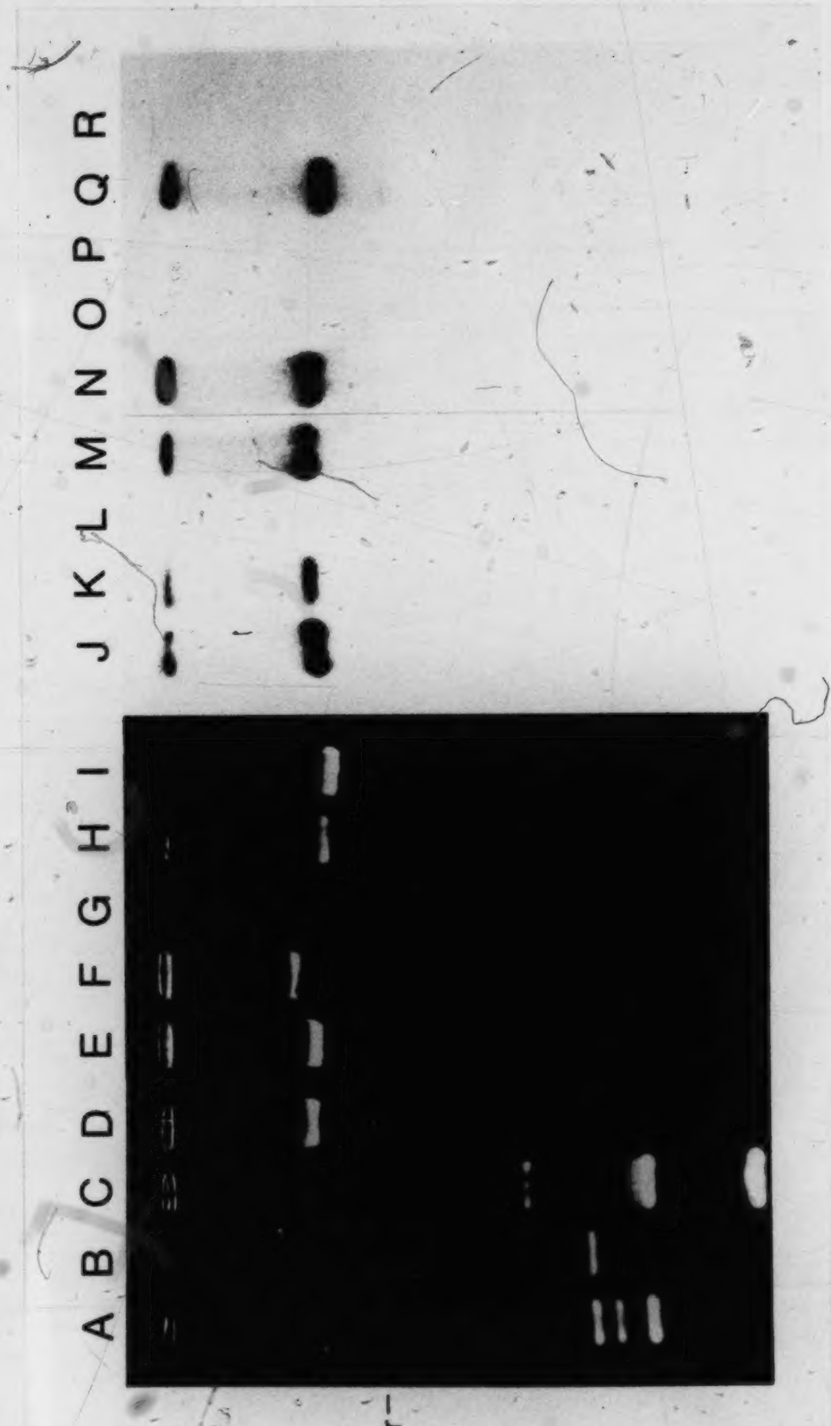
jejuni UA1. *C. coli* strain $25b_1$ showed two plasmid bands in agarose gels, one apparently larger than pUA466 and one apparently smaller (Figure 9.4, lane F). However, when the plasmids were purified on CsCl gradients, gel electrophoresis showed that only one plasmid $pLK25b_1$ was recovered. The band below the chromosome band probably represents the linear form of the large plasmid. *C. coli* strains 1a and 1b (Figure 9.4, lane A and B) contained 5 to 8 plasmid bands besides the 45 kb band.

The sizes of two Tc^R plasmids $pLK25b_1$ and $pLK29a_1$ were determined using gel electrophoresis and the standard curves obtained from *E. coli* plasmids of known molecular size. When TB buffer was used in gel electrophoresis, plasmids $pLK25b_1$ and $pLK29a_1$ appeared to have relative molecular sizes of 155 and 123 kb, respectively. When TA buffer was used, the relative sizes were 58 kb for $pLK25b_1$ and 45 kb for $pLK29a_1$.

To determine the homogeneity and location of the Tc^R determinant in the Tc^R isolates, the plasmids from all of the Tc^R isolates were hybridized separately with the 1.8 kb *Hinc*II fragment and the *tetM* probe. Some of the hybridization results with the 1.8 kb *Hinc*II probe are shown in Figure 9.4. Plasmids from Tc^R isolates which are of similar size to pUA466 hybridized with the 1.8 kb *Hinc*II fragment. For example, more than one plasmid is present in *C. coli* strains 1a and 1b (Figure 9.4, lanes A and B), but only one large plasmid hybridized with the 1.8 kb probe (Figure 9.4, lanes J and K). One of the plasmids in *C. coli*

Figure 9.4 Southern blot of plasmids from *Campylobacter* isolates hybridized with the 1.8 kb *Hinc*II fragment from pUA466.

Lanes A to I show the plasmid profile of *Campylobacter* strains. Lane A (1a) and B (1b) are Tc^R isolates from the same colon sample; lane C is Tc^S (2b) isolate, lane D (7a), E (9a$_1$) and F (25b$_1$) are Tc^R isolates from different colons; lane G is UA650, H is UA466 and I is UA649. The corresponding autoradiogram of lanes A to I with the 1.8 kb probe is shown on the right panel (lanes J to R).


A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
Chr—

2b (Figure 9.4, lane C) is of similar size to pUA466, but it did not hybridize with the 1.8 kb probe. The plasmid in Tc^R *C. coli* $25b_1$ (Figure 9.4,lane F) did not hybridize with the 1.8 kb probe (Figure 9.4, lane O). The hybridizations with the *tetM* probe gave similar results. Only the plasmids which hybridized with the 1.8 kb *Hin*cII fragment hybridized with the *tetM* probe.

9.3.5 Restriction endonuclease analysis and Southern transfer hybridization

Further characterization of the plasmids $pLK25a_1$ (from Tc^S *C. coli* $25a_1$), $pLK25b_1$ (from Tc^R *C. coli* $25b_1$) and $pLK29a_1$ (from Tc^R *C. coli* $29a_1$) was done by restriction digestions with endonucleases *Acc*I and *Hin*cII. pUA466 was included as a control. The average molecular sizes of the fragments obtained after digestion are summarized in Table 9.3. By adding up the sizes of the restriction fragments, $pLK25a_1$ (76 kb) is larger than $pLK25b_1$ (64 kb). The plasmid $pLK29a_1$ has a molecular size of 53 kb which is slightly larger than pUA466 (45 kb). The restriction digest patterns of $pLK25a_1$ and $pLK25b_1$ differed from that of pUA466. The 4.2 *Acc*I fragment was absent in $pLK25a_1$ and $pLK25b_1$ (see Figure 9.5). Moreover, the 1.8 kb *Hin*cII fragment was absent in both plasmids (see Figure 9.6). The restriction digestion patterns of $pLK29a_1$ with *Acc*I and *Hin*cII are shown in Figure 9.5 (lane D) and Figure 9.6 (lane E), respectively. When $pLK29a_1$ was digested with *Acc*I, there was a 4.4 kb fragment

Table 9.3 The restriction fragment sizes of plasmids in *Campylobacter* strains UA466, $25a_1$, $25b_1$ and $29a_1$.

Plasmid	Restriction endonuclease	Average fragment size kb	Total plasmid size kb
pUA466	*Acc*I	14.2, 13.8, 8.2, 4.2, 3.6	44.0
	*Hinc*II	19.0, 19.0, 6.2, 1.8	46.0
pLK25a_1	*Acc*I	26.6, 26.6, 13.1, 9.7	76.0
	*Hinc*II	45.0, 31.0	76.0
pLK25b_1	*Acc*I	41.0, 14.5, 10.0	65.5
	*Hinc*II	45.0, 16.5, 1.4	62.9
pLK29a_1	*Acc*I	34.0, 10.0, 4.4, 3.6	52.0
	*Hinc*II	31.0, 21.5, 2.0	54.5

Figure 9.5 The restriction fragment patterns of *Campylobacter* plasmids digested with *Acc*I and their Southern hybridization with the 1.8 kb *Hin*cII fragment probe.

The gel electrophoresis of *Acc*I digest of pUA466 (A), pLK25a_1, (B), pLK25b_1, (C), and pLK29a_1 (D) are shown on the left panel and the corresponding autoradiogram of pUA466 (E), pLK25a_1 (F), pLK25b_1 (G), and pLK29a_1 (H) is shown on the right panel.

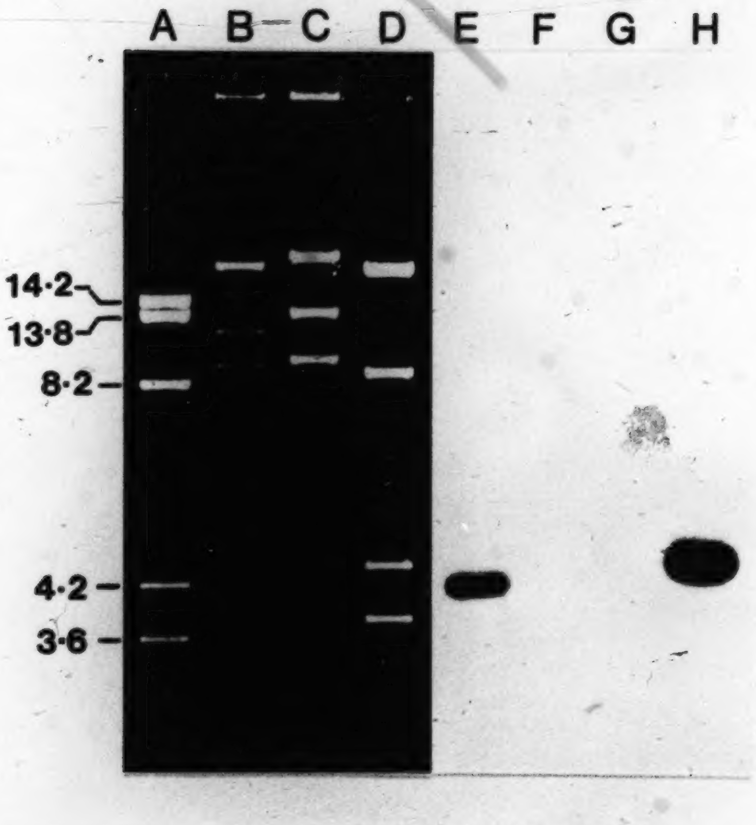
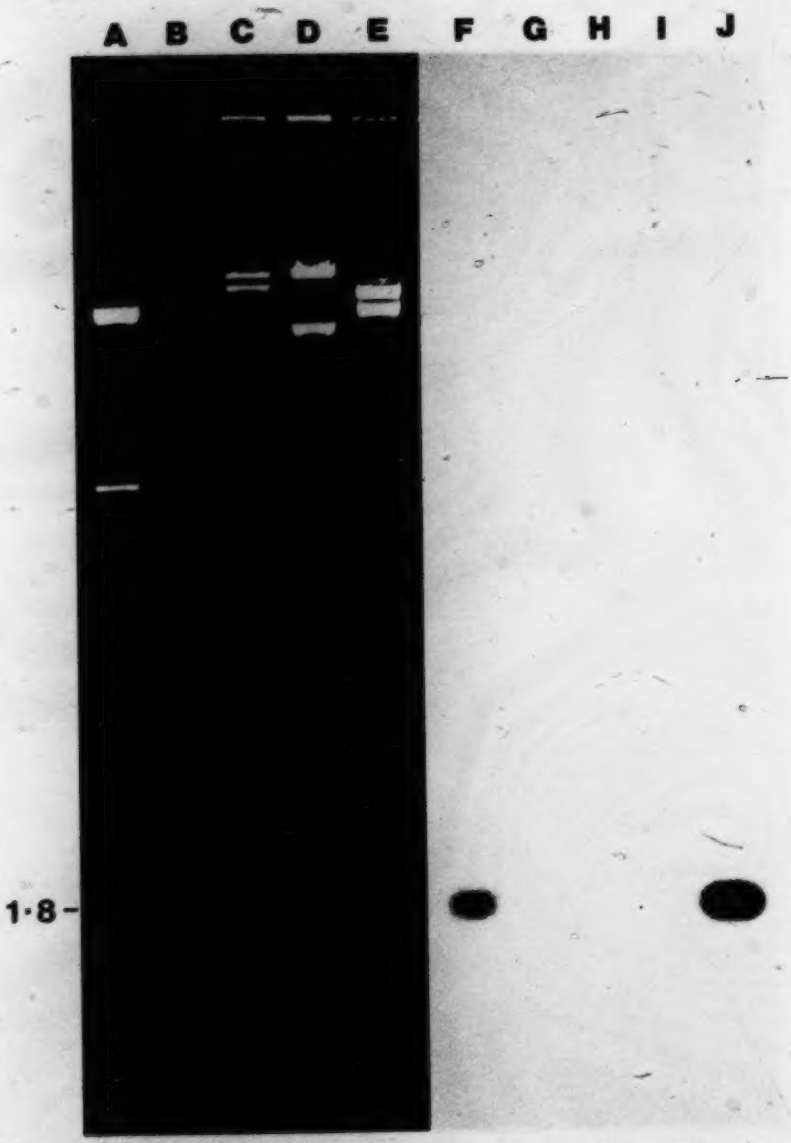

A
B
C
D
E
F
G
H
14·2
13·8
8·2
4·2
3·6

Figure 9.6 The restriction fragment pattern of *Campylobacter* plasmids digested with *Hinc*II and their Southern hybridization with the 1.8 kb *Hinc*II fragment probe. The gel electrophoresis of pUA466 (A), whole plasmid in 25b$_1$ (B), pLK25a$_1$ (C), pLK25b$_1$ (d) and pLK29a$_1$ (E) is shown on the left panel. The corresponding autoradiogram of pUA466 (F), whole plasmid in 25b$_1$ (G), pLK25a$_1$ (H), pLK25b$_1$ (I), and pLK29a$_1$ (J) is shown on the right panel.

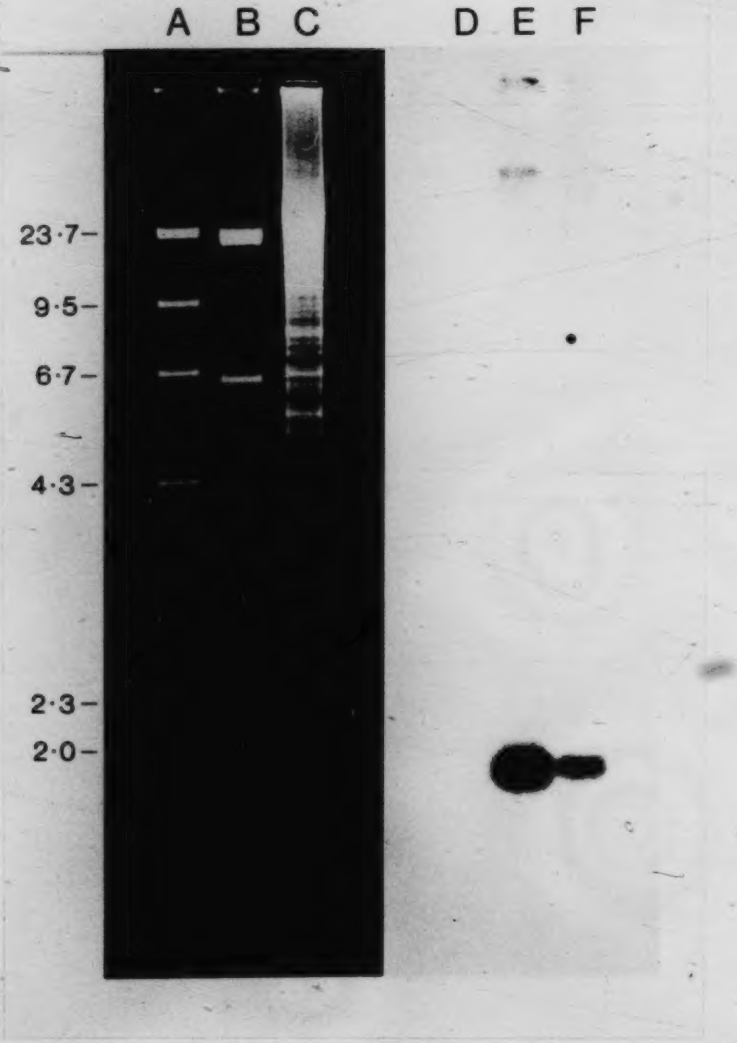

A
B
C
D
E
F
G
H
I
J
1·8

present that was similar in size to the 4.2 kb *Acc*I fragment of pUA466. A small fragment about 1.8 to 2.0 kb was present in pLK29a$_1$ when digested with *Hin*cII.

Southern transfer hybridization of the *Acc*I fragments of pLK25a$_1$, pLK25b$_1$ and pLK29a$_1$ with the 1.8 kb *Hin*cII fragment is shown in Figure 9.5. The 1.8 kb *Hin*cII fragment hybridized to the 4.2 kb *Acc*I fragment of pUA466 and the 4.4 kb *Acc*I fragment of pLK29a$_1$, but there was no hybridization to any fragments of pLK25a$_1$ and pLK25b$_1$. The 1.8 kb *Hin*cII fragment did not hybridize with the *Hin*cII fragments of plasmids pLK25a$_1$ and pLK25b$_1$ (Figure 9.6, lanes G and H). This confirms the results of earlier experiments that there is no homology between the 1.8 kb *Hin*cII fragment and pLK25a$_1$ and pLK25b$_1$.

Since the DNA from *C. coli* 25b$_1$ hybridized with the 1.8 kb *Hin*cII fragment with the dot blot experiment, but not in the plasmid, the probable location of the Tc^R determinant in this strain is on the chromosome. The chromosomal location of Tc^R in *C. coli* 25b$_1$ was confirmed by hybridization of the *Hin*cII digest of the chromosomal DNA of *C. coli* 25b$_1$ with the 1.8 kb *Hin*cII probe (see Figure 9.7). The 1.8 kb *Hin*cII probe was homologous with a 1.8 kb *Hin*cII fragment present in the chromosome of *C. coli* 25b$_1$. Therefore, *C. coli* 25b$_1$ contains the Tc^R determinant at a chromosomal site rather than on a plasmid.

Figure 9.7 The Southern blot hybridization of the total genomic DNA from $25b_1$ with the 1.8 kb *Hin*cII probe. The gel electrophoresis of the *Hin*cII digests of the plasmid pUA466 (lane B) and the total genomic DNA of $25b_1$ (lane C) is shown on the left panel. The corresponding autoradiogram of phage λ (D), pUA466 (E), and the total genomic DNA of $25b_1$ (F) is shown on the right panel. The molecular size standards of phage λ *Hin*dIII digest is shown in lane A and the sizes in kb are indicated in the left margin.


A
B
C
D
E
F
23·7–
9·5–
6·7–
4·3–
2·3–
2·0–

9.3.6 Southern blot hybridization of plasmids with pUA649

Since pUA649 (the Tc^S deletion derivative of pUA466) lacks most of the Tc^R determinant, it was used to assess the homology of plasmids present in *Campylobacter* strains. A series of plasmids from both Tc^S and Tc^R *Campylobacter* strains was hybridized with pUA649. The results are summarized in Table 9.4 and illustrated in Figure 9.8. In this study, all of the plasmids which hybridized with the pUA649 probe had sizes similar to pUA466 (see Figure 9.8). For example, one of the plasmids in *C. coli* strains 1a and 75a$_1$ was of similar size to pUA466 and it hybridized with pUA649 (Figure 9.8, lanes L and R). Plasmids in some of the Tc^S *Campylobacter* strains also showed positive hybridization with pUA649. For example, one of the three plasmids in Tc^S *C. coli* strain 64a (Figure 9.8, lane D) was similar in size to pUA466 and it hybridized with pUA649 (Figure 9.8, lane O).

9.3.7 Southern blot and dot blot hybridization of total genomic DNA with pUA649 probe

To determine if homology exists between the vector component of Tc^R plasmids and the chromosomal DNA, pUA649 was hybridized to the chromosome of *C. jejuni* UA650, a plasmidless derivative of *C. jejuni* UA466. The results are illustrated in the autoradiogram shown in Figure 9.9. Several chromosomal fragments of *C. jejuni* UA650 hybridized with pUA649. Therefore, apart from the Tc^R determinant,

Table 9.4 Southern transfer hybridization of plasmids from Tc^R and Tc^S *Campylobacter* isolates with pUA649.

Hybridization	Tetracycline susceptibility	Plasmid source
positive	Tc^R	UA466, 1a, $48a_1$, $75a_1$
	Tc^S	UA649, 16b, 21a, 64a
negative	Tc^R	$25b_1$
	Tc^S	UA650, $12a_7$, 72b*, 73b*, 78b, 76a, 82b

* *C. coli* strains 72b and 73b are clinical isolates obtained from L. Mueller, Alberta Provincial Laboratory of Public Health.

Figure 9.8 Southern blot hybridization of total plasmids from *Campylobacter* strains with pUA649.

The plasmid profiles of UA466 (A), 1a (B), 2b (C), $28a_1$ (D), $64a_1$ (E), 72b (F), 73b (G), $75a_1$ (H), 76a (I) and UA580 (J) are shown on the left panel. The corresponding autoradiogram of plasmids in UA466 (K), 1a (L), 2b (M), $28a_1$ (O), $64a_1$ (P), 72b (Q), 73b (R), $75a_1$ (S) and 76a (T) as shown on the right panel.

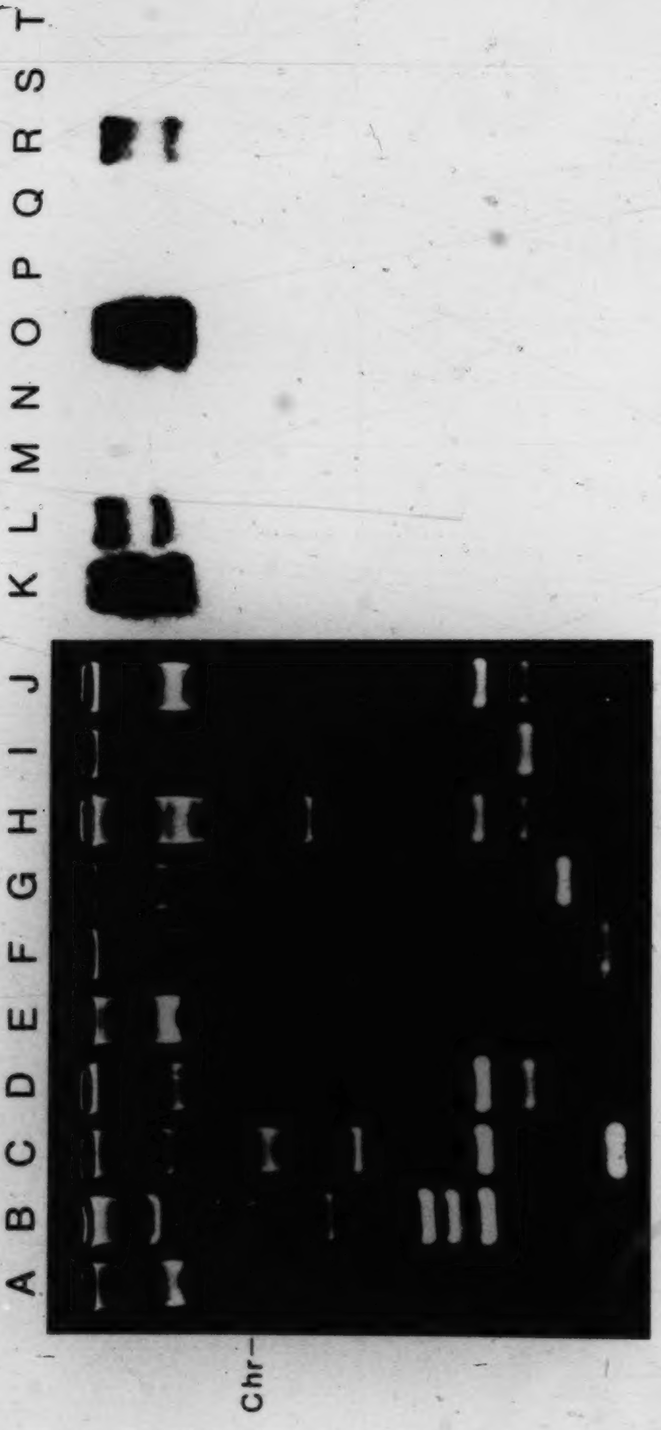
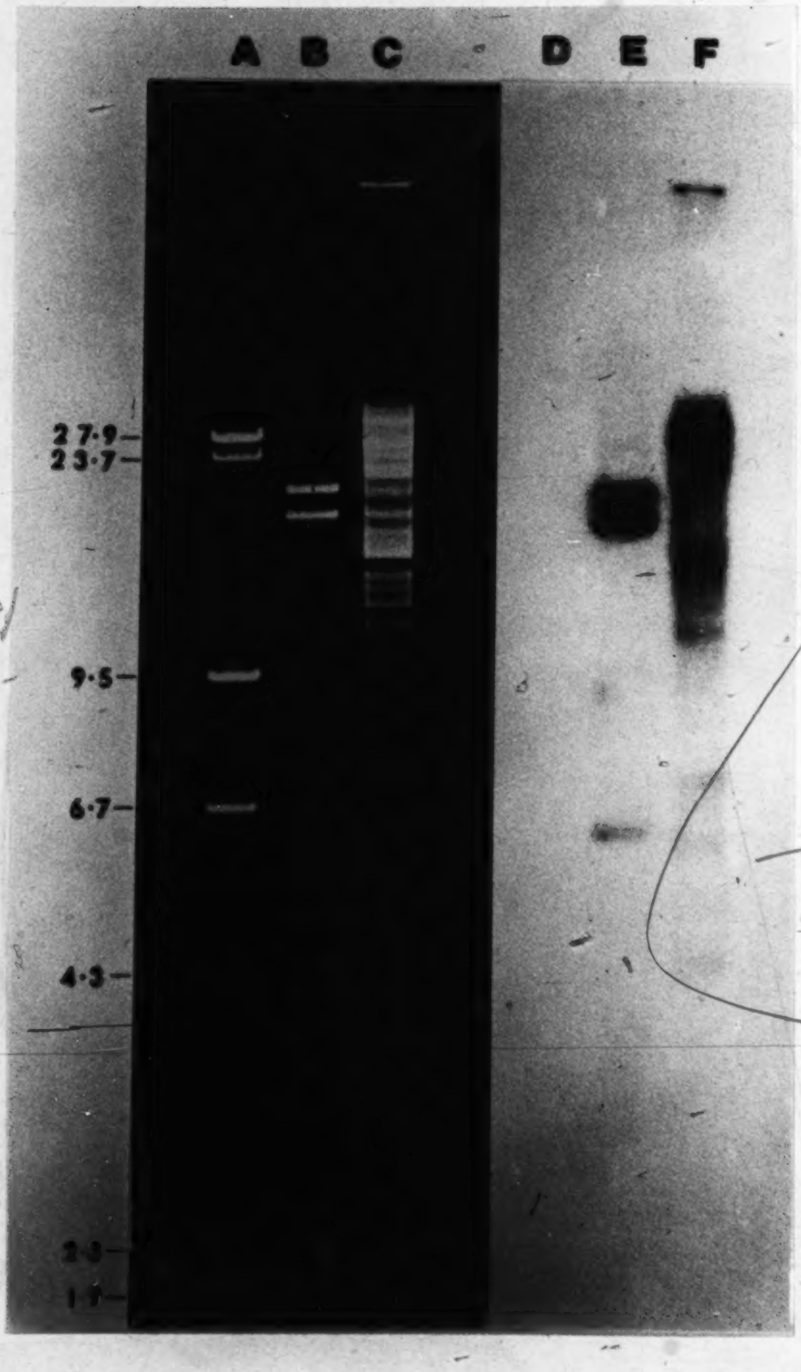

A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
Chr–

Figure 9.9 Hybridization of chromosomal DNA of *Campylobacter* digested with *Hin*cII to plasmid pUA649.

Lane A is *Hin*dIII digest of λ standard; lane B is *Hin*cII digest of pUA649 and lane C is *Hin*cII digest of chromosomal DNA of UA650. Lanes D, E and F shows the autoradiogram of lane A, B and C hybridized with ^{32}P-labelled pUA649.


A
B
C
D
E
F
27·9
23·7
9·5
6·7
4·3
2·3
1·9

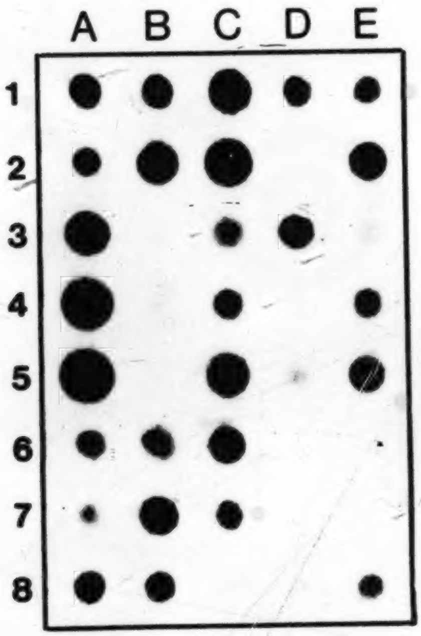
there is homology between the plasmid and chromosomal DNA.

Homology between the plasmid pUA649 and some Tc^S and Tc^R *Campylobacter* strains was determined using dot blot hybridization. The autoradiogram of the hybridization is shown in the left panel in Figure 9.10, and the identification grid of the autoradiogram is shown in the right panel. *C. jejuni* strains UA466 and UA649 (A3 and A4), as expected, hybridized with the pUA649 probe. UA650 (A5) and UA124 (A2), which are plasmidless derivatives of UA466 and UA1 also showed hybridization. Some other Tc^S *C. jejuni* strains (20a, 21a, 38b, NCTC11392 (UA579), NCTC 11168 (UA580) and UA67), and *C. coli* strains (28a and UA35) were also positive. *C. coli* NCTC 11353 (UA578) *C. fetus* subsp. *fetus* ATCC 27374 (UA60) and *C. laridis* UA487 did not hybridize with pUA649. Among the Tc^R isolates only *C. coli* $25b_1$ and *Campylobacter*-like strain $74a_1$ did not hybridize with the pUA649 probe.

9.3.8 Mating experiments

Mating experiments were conducted to confirm the Tc^R determinant is located on the chromosome and that it is not transferable from *C. coli* $25b_1$ to other *Campylobacter* strains. *C. coli* $25b_1$ was mated with *C. fetus* subsp. *fetus* UA60, *C. jejuni* UA67 and *C. laridis* UA487. No transconjugants were obtained. Occasionally, $25b_1$ mutated to nalidixic acid-resistant and colonies appeared on the selective plates when *C. fetus* subsp. *fetus* UA60 or

Figure 9.10 Autoradiogram prepared from dot blot hybridization of *Campylobacter* strains with pUA649. The autoradiogram is shown on the left panel and the corresponding grid with the culture number is on the right panel. Tetracycline-susceptible strains were indicated as T^S and the unmarked numbers are Tc^R.



	A	B	C	D	E
1	UA1	$9a_1$	$20a_3$ T^S	48a	UA35 T^S
2	UA124 T^S	64a T^S	21a T^S	$51a_1$ T^S	UA67 T^S
3	UA466	72b T^S	28a T^S	59b	UA578 T^S
4	UA649 T^S	73b T^S	29a	64b T^S	UA579 T^S
5	UA650 T^S	$74a_1$	38b T^S	76a T^S	UA580 T^S
6	1a	$76a_1$	34b	77b T^S	UA60 T^S
7	2b T^S	$12a_7$ T^S	44b	78a T^S	UA487 T^S
8	7a	16a T^S	$25b_1$	$82a_1$	UA124 T^S

nalidixic acid-resistant *C. jejuni* UA67 were used as recipients. These colonies were shown to be the donors by testing for hippurate hydrolysis when UA67 was used as the recipient, and for cephalothin resistance when UA60 was used as the recipient. *C. coli* $25b_1$ is cephalothin-susceptible and hippurate negative and it can therefore, be distinguished from UA60 and UA67.

9.3.9 Curing experiment

Attempts to eliminate plasmids from *C. coli* $25b_1$ using ethidium bromide were unsuccessful, although curing experiments were repeated three times. Moreover, no Tc^S colonies were obtained. A total of 144 Tc^R colonies was checked for plasmid content, and all of them had the same plasmid profile as the parent strain.

9.4 Discussion

In this study, some (6.7%) of the colons tested yielded Tc^R *Campylobacter* strains. Four of these colons were from animals that had not been exposed to antibiotics. The animals probably acquired the Tc^R strains from the environment. In studies conducted on chicken farms, it was shown that resistant enteric strains could spread from chickens fed with antibiotic-containing feeds, to chickens without antibiotics, and then to human handlers (Levy, 1984). In fact, ever since the introduction of the tetracycline antibiotics in 1947, Tc^R strains of bacteria have been encountered at an increasing rate and they have traversed many genera (Levy, 1976). This is probably due to the use of tetracycline as a growth promotant in animal feeds and also its frequent use for treatment of bacterial infections (Levy, 1984).

C. coli 48a, 48b and 59b were less resistant to tetracycline than other reported strains with plasmid encoded Tc^R (Taylor *et al.*, 1986). However, MICs below 16 µg/mL were not observed. Low levels of Tc^R in *Campylobacter* strains are often not plasmid-mediated (Taylor, 1981). Previous studies on *Campylobacter* plasmids have demonstrated (Taylor *et al.*, 1983a; Tenover *et al.*, 1985) that there is a high degree of DNA homology among the plasmids in *Campylobacter* spp. In this study, the Tc^R plasmid pUA466 was related to plasmids of similar sizes (about 40-50 kb). Some of these plasmids do not encode Tc^R, and it is possible that these plasmids lost the Tc^R determinant as observed with pUA649 (Taylor, 1986). Alternately, *Campylobacter* strains may carry an indigenous plasmid of 40-50 kb, which subsequently acquired the tetracycline resistance determinant from another source.

Although a wide range of sizes (42 to 100 kb) have been reported for *Campylobacter* Tc^R plasmids (Tenover *et al.*, 1985), Tc^R plasmids greater than 60 kb were not observed in this study. Comparison of plasmid sizes studied in different laboratories is difficult because of the use of different conditions of plasmid isolation and gel electrophoresis

(Taylor *et al.*, 1981; Tenover *et al.*, 1983). Depending of the conditions of plasmid isolation and gel electrophoresis, the relative size of plasmids may not be accurate, especially if plasmids in *E. coli* were used as molecular size standards. This may due to different degrees of supercoiling (Gellert, 1981) and the bending of DNA (Koo *et al.*, 1986; Wu and Crothers, 1984). Plasmids may also exist in different forms, linear, open or closed circles (Hintermann *et al.*, 1981). In this study, three plasmids pLK25a$_1$, pLK25b$_1$ and pLK29a$_1$ were sized using both whole plasmid and restriction digestion gel electrophoresis. The sizes estimated from standard curves using plasmids of known size, obtained by gel electrophoresis in TB buffer, appeared to be much larger than estimates obtained using TA buffer. Based on our unpublished data on sizing of plasmids using electron microscopy and gel electrophoresis, plasmid sizes obtained by gel electrophoresis of restriction digests would be accurate. The size estimated from standard curves using gel electrophoresis of whole plasmids in TA buffer was closer to the estimates obtained from electron microscopy than TB buffer. The Tc^R plasmid pMAK175 was first estimated to be 38 Mdal or 58.5 kb (Taylor *et al.*, 1981) using *E. coli* plasmids of known sizes as standards in gel electrophoresis. Subsequently, this plasmid was estimated to be 29 Mdal or 45 kb using electron microscopy and by adding the sizes of restriction fragments (Taylor *et al.*, 1983a).

Based on the hybridization study using the pUA649 probe and total genomic DNA from Tc^S and Tc^R strains, the Tc^R plasmid (pUA466) showed homology to chromosomal DNA. Therefore, it is not appropriate to use the entire plasmid as a DNA probe to detect Tc^R strains or to study the diversity of Tc^R in this genus. Since pUA649 lacks the 1.8 kb *Hin*cII fragment, the homology is probably not due to the Tc^R, but rather due to the interchange of DNA between chromosome and plasmids. The G+C content of the Tc^R plasmid pMAK175 is 32.5% and was similar to that found in the *C. jejuni* chromosome (Taylor *et al.*, 1983a). This suggests that the tetracycline vector plasmid may have arisen in this species.

The 1.8 kb *Hin*cII fragment from pUA466 is highly specific for the Tc^R in *Campylobacter* and can be used as a probe with sensitivity similar to *E. coli* DNA probes for heat-labile and heat-stable toxins (Moseley *et al.*, 1982). Only 10^5 to 10^6 cells are required to detect Tc^R. In this study, no false positives or negatives were encountered. The 1.8 kb *Hin*cII fragment is located within the tetracycline-resistant determinant (Taylor, 1986). This cloned fragment is useful for studying *Campylobacter* Tc^R. The 5 kb *tetM* DNA probe from pJI3 in *S. agalactiae* has homology with the 1.8 kb *Hin*cII fragment from pUA466 (Taylor, 1986). When the 5 kb *tetM* probe is used, Tc^R strains can also be detected. However, the *tetM* probe has a much lower sensitivity and requires a lower stringency of hybridization (37°C with 50%

formamide). This is possibly due to the larger size of the probe and also the lower degree of homology between the *Campylobacter* and streptococcal Tc^R determinants. The *tetM* probe showed hybridization with the Tc^S deletion derivative UA649, indicating that false positives may be encountered if deletion mutants are found in the natural environment. Although the streptococcal *tetM* determinant has been sequenced (Martin *et al.*, 1986), the sequence of the *Campylobacter* Tc^R determinants is not yet available, so that the degree of homology and the function of the homologous region(s) is not clear. Therefore, it is not possible to decide unequivocally if the Campylobacter Tc^R determinant was acquired from a gram-positive coccus. For detection purposes, both probes may be used. However, for classification or study of the diversity of the Tc^R in the genus *Campylobacter* it would be more appropriate to use the 1.8 kb *Hin*cII fragment in hybridization experiments.

In this study, all the Tc^R *C. coli* and *C. jejuni* strains hybridized with the 1.8 kb *Hin*cII fragment, therefore, they probably have the same mechanism of resistance. Although the Tc^R determinant is usually encoded on a plasmid, occasionally it could be chromosomally-mediated as shown in *C. coli* 25b$_1$. Chromosomally-mediated high levels of Tc^R in *Campylobacter* have not been reported previously.

9.5 Bibliography

Austen, R. A., and T. J. Trust. 1980. Detection of plasmids in the related group of the genus *Campylobacter*. FEMS Microbiol. Lett. 8:201-204.

Birnboim, H. C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7:1513-1523.

Bradbury, W. C., M. A. Marko, J. N. Hennessy, and J. L. Penner. 1983. Occurrence of plasmid DNA in serologically defined strains of *Campylobacter jejuni* and *Campylobacter coli*. Infect. Immun. 40:460-463.

Bradbury, W. C., and D. L. G. Munroe. 1985. Occurrence of plasmids and antibiotic resistance among *Campylobacter jejuni* and *Campylobacter coli* isolated from healthy and diarrheic animals. J. Clin. Microbiol. 22:339-346.

Burdett, V., J. Inamine, and S. Rajagopalan. 1982. Heterogeneity of tetracycline resistance determinants in *Streptococcus*. J. Bacteriol. 149:995-1004.

Dretzen, G., M. Bellard, P. Sassone-Corsi, and P. Chambon. 1981. A reliable method for the recovery of DNA fragments from agarose and acrylamide gels. Anal. Biochem. 112:295-298.

Gellert, M. 1981. DNA topoisomerases. Annu. Rev. Biochem. 50:879-910.

Hintermann, G. H.-M. Fischer, R. Crameri, and R. Hutter. 1981. Simple procedure for distinguishing CCC, OC, and L forms of plasmid DNA by agarose gel electrophoresis. Plasmid 5:371-373.

Koo, H.-S., H.-M. Wu, and D. M. Crothers. 1986. DNA bending at adenine-thymine tracts. Nature 320:501-506.

Lambert, T., G. Gerbaud, P. Trieu-Cuot, and P. Courvalin. 1985. Structural relationship between the genes encoding 3'-aminoglycoside phosphotransferases in *Campylobacter* and in gram-positive cocci. Ann. Inst. Pasteur 136B:135-150.

Levy, S. B. 1984. Resistance to the tetracyclines, p. 191-240. *In* L. E. Bryan (ed.), Antimicrobial drug resistance. Academic Press, Inc., N.Y.

Levy, S. B., G. B. FitzGerald, and A. B. Macone. 1976. Changes in intestinal flora of farm personnel after introduction of a tetracycline-supplemented feed on a farm. N. Engl. J. Med. 295:583-588.

Maniatis, T., A. Jeffrey, and D. G. Kleid. 1975. Nucleotide sequence of the rightward operator of phage lambda. Proc. Natl. Acad. Sci. U.S.A. 72:1184-1188.

Marmur, J., and P. Doty. 1961. Thermal renaturation of deoxyribonucleic acids. J. Mol. Biol. 3:585-594.

Martin, P., P. Trieu-Cuot, and P. Courvalin. 1986. Nucleotide sequence of the *tetM* tetracycline resistance determinant of the streptococcal conjugative shuttle transposon Tn*1545*. Nucleic Acids Res. 14:7047-7058.

McConaughy, B. L., C. D. Laird, and B. J. McCarthy. 1969. Nucleic Acid reassociation in formamide. Biochem. 8:3289-3294.

Mendez, B., C. Tachibana, and S. B. Levy. 1980. Heterogeneity of tetracycline resistance determinants. Plasmid 3:99-108.

Moseley, S. L., P. Echeverria, J. Serriwatana, C. Tirapat, W. Chaicumpa, T. Sakuldaipeara, and S. Falkow. 1982. Identification of enterotoxigenic *Escherichia coli* by colony hybridization using three enterotoxin gene probes. J. Infect. Dis. 145:863-869.

Ng, L.-K., M. E. Stiles, and D. E. Taylor. 1985. Inhibition of *Campylobacter coli* and *Campylobacter jejuni* by antibiotics used in selective growth media. J. Clin. Microbiol. 22:510-514.

Portnoy, D. A., S. L. Moseley, and S. Falkow. 1981. Characterization of plasmids and plasmid-associated determinants of *Yersinia enterocolitica* pathogenesis. Infect. Immun. 31:775-782.

Roberts, M. C., and G. E. Kenny. 1986. Dissemination of the *tetM* tetracycline resistance determinant to *Ureaplasma urealyticum*. Antimicrob. Agent Chemother. 29:350-352.

Schildkraut, C., and S. Lifson. 1965. Dependence of the melting temperature of DNA on salt concentration. Biopolymers 3:195-208.

Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

Taylor, D. E. 1981. *Campylobacter jejuni*: Characteristic features of the organism and identification of transmissible plasmids in tetracycline-resistant clinical isolates, p.61-70. *In* S. B. Levy, R. C. Clowes, and E. L. Koenig (ed.) Molecular biology, pathogenicity, and ecology of bacterial plasmids. Plenum Publishing Corp.

Taylor, D. E. 1986. Plasmid-mediated tetracycline resistance in *Campylobacter jejuni*: Expression in *Escherichia coli* and identification of homology with Streptococcal class M determinant. J. Bacteriol. 165:1037-1039.

Taylor, D. E., and E. Brose. 1985. Restriction endonuclease mapping of R27 (TP117), an incompatibility group HI subgroup1 plasmid from *Salmonella typhimurium*. Plasmid 13:75-77.

Taylor, D. E., N. Chang, R. S. Garner, R. Sherburne, and L. Mueller. 1986. Incidence of antibiotic resistance and characterization of plasmids in *Campylobacter jejuni* strains isolated from clinical sources in Alberta, Canada. Can. J. Microbiol. 32:28-32.

Taylor, D. E., S. A. DeGrandis, M. A. Karmali, and P. C. Fleming. 1981. Transmissible plasmids from *Campylobacter jejuni*. Antimicrob. Agents Chemother. 19:831-835.

Taylor, D. E., R. S. Garner, and B. J. Allan. 1983a. Characterization of tetracycline resistance plasmids from *Campylobacter jejuni* and *Campylobacter coli*. Antimicrob. Agents Chemother. 24:930-935.

Taylor, D. E., D. G. Newell, and A. D. Pearson. 1983b. Incidence of plasmid DNA in strains of *Campylobacter jejuni* isolated from stool specimens at 37 C and 43 C. J. Infect. Dis. 147:965-966.

Tenover, F. C., M. A. Bronsdon, K. P. Gordan, and J. J. Plorde. 1983. Isolation of plasmids encoding tetracycline resistance from *Campylobacter jejuni* strains isolated from simians. Antimicrob. Agents Chemother. 23:320-322.

Tenover, F. C., S. Williams, K. P. Gordon, C. Nolan, and J. J. Plorde. 1985. Survey of plasmids and resistance factors in *Campylobacter jejuni* and *Campylobacter coli*. Antimicrob. Agents Chemother. 27:37-41.

Wu, H.-M., and D. M. Crothers. 1984. The locus of sequence-directed and protein-induced DNA bending.

Nature 308:509-513.

10. Discussion and Conclusions

Electron microscopic studies of *Campylobacter coli* and *Campylobacter jejuni* showed that cells from both species are morphologically similar and that they undergo morphological changes as the cells age. The young or actively growing cells are spiral-shaped and older cells are coccoid-shaped. This phenomenon has been documented elsewhere (Smibert, 1978). In addition, a "donut" form, not previously described was shown to be a possible intermediate between the spiral and the coccus-shaped cells. The correlation between the microscopic count and the plate count of cell suspensions demonstrated that the coccus forms are not viable. When *C. coli* and *C. jejuni* were grown in broth medium for 24 to 48 h, the cells were mainly spiral-shaped, as a result, 24 to 48-h broth cultures were used for quantitative studies.

With the discovery of *Campylobacter* and their significance in human infections, many new media were proposed for their isolation from clinical specimens. Different basal media were used in the selective media for isolation and growth of *C. jejuni* and *C. coli*. Most of the basal media require growth supplements such as animal blood and/or FBP (a combination of ferrous sulfate, sodium metabisulfite and sodium pyruvate). Animal blood is not a well-defined supplement, therefore, it would be advantageous to use a blood-free medium for the growth of *C. jejuni* and *C. coli*. By comparing the growth characteristics of *C. coli* and *C. jejuni* on eight basal media, three media without added supplements (brucella agar, campylobacter agar base and Mueller-Hinton (agar) were shown to be suitable for the growth of *Campylobacter* cells in pure culture. Mueller-Hinton agar is recommended and widely used for antibiotic susceptibility testing (Barry, 1980), therefore, it was chosen as a basal medium for the growth of *C. coli* and *C. jejuni* in these studies.

The development of media for the isolation of *Campylobacter* associated with gastroenteritis was based on the antibiotic susceptibility of "*C. jejuni*". It was assumed that these media are suitable for the growth of *C. coli*. There are many reports of the antibiograms of *C. jejuni* (see review by Vanhoof, 1984), however, there is less information on the antibiotic susceptibility for *C. coli*. As a result, the MIC of the antibiotics used in selective media for laboratory and fresh isolates of *C. coli* and *C. jejuni*, as well as the inhibitory effect of the selective media on these strains, were determined. Of the *C. coli* strains tested, the MICs of trimethoprim, vancomycin and bacitracin were the same as those obtained for *C. jejuni*, however, the MICs of rifampicin, cephalothin, novobiocin and colistin varied over a greater range than *C. jejuni* strains tested.

Susceptibility of three *C. jejuni* and seven *C. coli* laboratory strains to combinations of antibiotics used in selective media was compared using a plate count method. *C. jejuni* grew well in the presence of six out of the nine antibiotic combinations tested. The other three combinations caused a 1.5- to 5-log cycle reduction of *C. jejuni* counts. In contrast, only one out of the nine antibiotic combinations showed no significant inhibitory effect for the seven *C. coli* strains tested. The other six *C. coli* strains were inhibited by two to six of the antibiotic combinations. Hence, the *C. coli* strains tested were more susceptible to antibiotics in selective media than the *C. jejuni* strains. The antibiotic susceptibilities of laboratory strains may differ from organisms in their natural habitat (Davies and Smith, 1978). To confirm that *C. coli* strains are indeed more susceptible than to *C. jejuni* antibiotics used in selective media, fresh isolates of *C. coli* and *C. jejuni* were isolated from swine and cattle colons.

Based on the MICs of fresh isolates to a range of antibiotics used in selective media, twelve *C. coli* and six *C. jejuni* strains were selected and the inhibitory effect of antibiotic combinations on these isolates was compared. Of the 12 strains of *C. coli* tested, 10 strains were inhibited by at least three of the eleven media included in the study. Only one strain of *C. jejuni* was inhibited on one medium. Therefore, fresh isolates of *C. coli* gave similar results to those found in laboratory adapted strains, that is, they were more susceptible than *C. jejuni* to antibiotic selective media. Although media containing cefoperazone (Hutchinson and Bolton, 1984; Karmali *et al.*, 1986) had no inhibitory effect on most *C. coli* and *C. jejuni* strains, the cephalothin-susceptible *C. coli* strains were inhibited on these media. In these studies of the inhibitory effects of selective media, actively growing *Campylobacter* cells were used. In food and environmental samples, *C. coli* may be stressed or injured, therefore, the isolation of these organisms using antibiotics as selective agents would be even more difficult (Hanninen, 1982; Humphrey and Cruickshank, 1985; Ray and Johnson, 1983). If low numbers of *C. coli* are present in stool samples, it has been reported that *C. coli* may not be detected (Hodge and Terro, 1984).

In addition to the occurrence of cephalothin-susceptible strains of *C. coli* (Brooks *et al.*, 1986; Karmali and Skirrow, 1984), other atypical characteristics of *C. coli* and *C. jejuni* have been reported. These include the occurrence of nalidixic acid-resistant strains (Taylor *et al.*, 1985) and hippurate-negative strains (Roop *et al.*; 1984; Totten *et al.*, 1985). In our study, *Campylobacter* strains with MICs of ≤64 µg of cephalothin per mL were encountered. Some of these strains were confirmed to be *C. coli* using DNA homology determinations. At present, there are very few phenotypic characteristics for differentiating *Campylobacter* spp. DNA homology studies give a definitive method of confirming *Campylobacter* organisms at the species level. To simplify the procedure for DNA homology determination, a dot blot hybridization method was developed, which eliminates the requirement for isolation of pure DNA for the slot blot hybridization of *Campylobacter* spp.

For strain differentiation of *C. coli* and *C. jejuni* for epidemiological studies, biotyping (Hébert, *et al*., 1982; Lior, 1984; Skirrow and Benjamin, 1980), antibiograms (Bopp *et al*., 1985), and plasmid profiles (Bopp *et al*., 1985) have been used. These parameters were used in this study to differentiate the *Campylobacter* strains from swine and cattle. Most of the *C. coli* and *C. jejuni* strains belong to biotype I, therefore, biotyping (Lior, 1984) alone is not adequate to differentiate these strains. However, with the additional information on antibiotic resistance, especially tetracycline-resistance (Tc^R) and plasmid profiles, the *Campylobacter* strains could be identified more effectively.

Tetracycline-resistant strains of *C. coli* and *C. jejuni* were isolated from animals that had not been previously exposed to antibiotics. Therefore, the Tc^R strains were selected for further study to determine the homogeneity of the Tc^R mechanisms among these strains and the clinical isolates. To study the Tc^R in these strains, DNA probes were used. The results indicated that the Tc^R mechanisms among the *Campylobacter* strains were similar. Using a DNA probe, the chromosomal location of the Tc^R determinant was identified. In the past, Tc^R determinants were only found on plasmids (Taylor *et al*., 1986). The method used in our study serves as a model for the development and hybridization of other DNA probes for diagnostic or detection purposes.

These studies showed that *C. coli* and *C. jejuni* are similar morphologically and biochemically, but different in susceptibilities to antibiotics used in selective media. This probably accounts for the lower incidence of gastroenteritis caused by *C. coli*. The differentiation of *C. jejuni* and *C. coli* relies solely on the hippurate hydrolysis test, and DNA homology is the only definitive method for confirming their identity. The failure to differentiate these two species may also affect the epidemiological data. The development of more reliable methods of detection or isolation of *C. coli* are needed. The use of DNA probes or monoclonal antibody techniques that could eliminate the cultural step would be advantageous.

10.1 Bibliography

Barry, A. L. 1980. Procedure for testing antibiotics in agar media: theoretical considerations, p. 1-23. *In* V. Lorian (ed.), Antibiotics in laboratory medicine. The William and Wilkins Co., Baltimore.

Bopp, C. A., K. A. Birkness, I. K. Wachsmuth, and T. J. Barrett. 1985. In vitro antimicrobial susceptibility, plasmid analysis, and serotyping of epidemic-associated *Campylobacter jejuni*. J. Clin. Microbiol. 21:4-7.

Brooks, B. W., M. M. Garcia, A. D. E. Fraser, H. Lior, R. B. Stewart, and A. M. Lammerding. 1986. Isolation and characterization of cephalothin-susceptible *Campylobacter coli* from slaughter cattle. J. Clin. Microbiol. 24:591-595.

Davies, J., and D. I. Smith. 1978. Plasmid-determined resistance to antimicrobial agents. Annu. Rev. Microbiol. 32:469-518.

Hanninen, M.-L. 1982. Effect of recovery medium on the isolation of *Campylobacter jejuni* before and after heat treatment. Acta. Vet. Scand. 23:416-424.

Hébert, G. A., D. G. Hollis, R. E. Weaver, M. A. Lambert, M. J. Blaser, and C. W. Moss. 1982. 30 years of campylobacters: Biochemical characteristics and a biotyping proposal for *Campylobacter jejuni*. J. Clin. Microbiol. 15:1065-1073.

Hodge, D. S., and R. Terro. 1984. Comparative efficacy of liquid enrichment medium for isolation of *Campylobacter jejuni*. J. Clin. Microbiol. 19:434.

Humphrey, T. J., and J. G. Cruickshank. 1985. Antibiotic and deoxycholate resistance in *Campylobacter jejuni* following freezing or heating. J. Appl. Bacteriol. 53:65-71.

Hutchinson, D. N., and F. J. Bolton. 1984. Improved blood free selective medium for the isolation of *Campylobacter jejuni* from faecal specimens. J. Clin. Pathol. 37:956-957.

Karmali, M. A., A. E. Simor, M. Roscoe, P. C. Fleming, S. S. Smith, and J. Lane. 1986. Evaluation of a blood-free, charcoal-based, selective medium for the isolation of *Campylobacter* organisms from feces. J. Clin. Microbiol. 23:456-459.

Karmali, M. A., and M. B. Skirrow. 1984. Taxonomy of the genus *Campylobacter*, p. 1-20. *In* J. P. Butzler (ed.), Campylobacter infection in man and animals. CRC Press, Inc., Boca Raton, Fla.

Lior, H., 1984. New, extended biotyping scheme for *Campylobacter jejuni*, *Campylobacter coli* and "*Campylobacter laridis*". J. Clin. Microbiol. 20:636-640.

Ray, B., and C. Johnson. 1983. Growth requirements of freeze-stressed *Campylobacter jejuni*. Abstr. Annu. Meet. Am. Soc. Microbiol., New Orleans, p.257.

Roop, R. M., II, R. M. Smibert, and N. R. Krieg. 1984. Improved biotyping schemes for *Campylobacter jejuni* and *Campylobacter coli*. J. Clin. Microbiol. 20:990-992.

Skirrow, M. B., and J. Benjamin. 1980. Differentiation of enteropathogenic campylobacter. J. Clin. Pathol. 33:1122.

Smibert, R. M. 1978. The genus *Campylobacter*. Annu. Rev. Microbiol. 32:673-709.

Taylor, D. E., N. Chang, R. S. Garner, R. Sherburne, and L. Mueller. 1986. Incidence of antibiotic resistance and characterization of plasmids in *Campylobacter jejuni* strains isolated from clinical sources in Alberta, Canada. Can. J. Microbiol. 32:28-32.

Taylor, D. E., L.-K. Ng, and H. Lior. 1985. Susceptibility of *Campylobacter* species to nalidixic acid, enoxacin, and other DNA gyrase inhibitors. Antimicrob. Agents Chemother. 28:708-710.

Totten, P. A., F. C. Tenover, K. C. S. Chen, K. L. Bruch, K. K. Holmes, and W. E. Stamm. 1985. Rapid genetic grouping test for identification of *Campylobacter* species, p. 234. *In* A. D. Pearson, M. B. Skirrow, H. Lior, and B. Rowe (ed.), Campylobacter III: Proceedings of the third international workshop on campylobacter infections, Ottawa. Public Health Laboratory Service, London.

Vanhoof, R. 1984. Susceptibility of campylobacters to antimicrobial agents, p.77-85. *In* J. P. Butzler (ed.), Campylobacter infection in man and animals. CRC Press, Inc., Boca Raton, Fla.